Toxicity of the antitumor drug mitoxantrone in pediatric and adult mice

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Dissertação de Candidatura ao grau de Mestre em Bioquímica da Universidade do Porto

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ABSTRACT

Anticancer therapy has evolved greatly with the introduction of new drugs that increase life expectancy. However, the increased efficacy leads to several side effects and damage, especially for non-cancer tissues, thus compromising the survival or the quality of life of the treated-patients. Mitoxantrone (MTX) is an anticancer agent widely used and one of its major adverse effects is cardiac toxicity that can affect up to 18% of MTX-treated patients. One of the most concerning MTX-cardiac side effects is heart failure that may be detected only years after cancer therapy cessation. Although MTX has a toxic clinical profile similar to doxorubicin, their mechanisms of cardiotoxicity differ and MTX mechanisms of cardiotoxicity remain largely unknown. MTX is also used in pediatric cancer and in multiple sclerosis in children. Children that survive cancer potentially have a long life-expectancy after treatment, but little is known about the overall risks of this population to develop heart toxicity after MTX.

Therefore, the present study aimed to evaluate the parameters and risk factors associated to MTX-cardiac toxicity as well as the toxicity towards the liver and kidneys, in pediatric (3 weeks) and adult (8-10 weeks) male CD-1 mice. Total cumulative doses of 4.5 mg/kg and 9.0 mg/kg of MTX were given as a result of 6 intraperitoneal injections (2 *per* week) in experiment 1. Cumulative doses of 7.0 mg/kg and 9.0 mg/kg MTX were used in experiment 2. This multiple administration procedure was used in order to mimic the human MTX therapy that comprises multiple MTX-administration in different cycles. After MTX administration, the animals were maintained, before sacrifice, in a drug-free period to allow the development of cumulative toxicity. In experiment 1, the surviving animals were sacrificed at day 20; in experiment 2, the 9.0 mg/kg MTX-treated animals were sacrificed one day after the last administration, whereas the 7.0 mg/kg MTX-treated animals were sacrificed 14 days after the last administration. The consumption of food and water, and animal weight were recorded along the experiments. After sacrifice, the following parameters were evaluated: total glutathione (GSHt), oxidized glutathione (GSSG), lipid peroxidation and adenosine triphosphate (ATP) on heart, liver, and kidney; cardiac apoptosis and histopathological examination; and finally aspartate aminotransferase (AST), alanine aminotransferase (ALT), total-creatine kinase (total-CK) and creatine-kinase MB (CK-MB) levels in the plasma.

In the studies developed in the present dissertation, pediatric population revealed itself as more resistant to MTX-induced toxicity as, in experiment 1, some of these animals survived with the 9.0 mg/kg cumulative MTX dose. In both experiments, the average body weight of pediatric mice almost did not vary with time, oppositely to what happened with adults. Elevation of plasma AST in the survivors of 9.0 mg/kg MTX-treated animals in experiment 1 may indicate heart damage since no changes were seen in ALT. The decreases in AST and ALT in 4.5 mg/kg MTX-treated adults may be related to their

significant body weight decrease or to other metabolic impairments. CK-MB levels in pediatrics treated with the cumulative dose of 7.0 mg/kg MTX were increased, while in the 9.0 mg MTX/kg mice, they were decreased, which reveals that the plasma concentration of this parameter is sensitive to altered physiological conditions. Lipid peroxidation was not altered in any groups and organs, excepting in the liver of 9.0 mg/kg MTX-treated pediatric mice in experiment 2, where it was decreased. In experiment 1, the evaluation of glutathione contents in the heart showed an increase in the GSH/GSSG ratio in the 9.0 mg/kg-treated pediatric mice indicating that they may have adapted their antioxidant defense levels over time. In fact, when the pediatric population was sacrificed 24h after the last MTX administration in the same cumulative dose, no changes were observed. Oppositely, the 9.0 mg/kg MTX-treated adults in experiment 2 had higher values of cardiac GSSG. In the heart of the 7.0 mg/kg MTX-treated animals, no significant changes were observed in the adults, whereas in the pediatric population GSHt levels increased. In this pediatric group, GSSG levels had a slight but not significant increase whereas no alteration in the GSH/GSSG ratio was seen. Therefore, the adaptation mechanisms on the GSH pathways did not occurred in this lower cumulative concentration (and less elapsed time) in pediatrics when compared to the 9.0 mg/kg dose in experiment 1. The liver of 9.0 mg/kg MTX-treated pediatrics showed low GSH/GSSG ratio in experiment 1, thus the heart and liver had different responses towards MTX. However and still regarding the liver, adults of 4.5 mg/kg MTX dose had an increased GSH/GSSG ratio reflecting a possible activation of compensatory mechanisms by increased GSH synthesis towards the MTX-induced hepatic damage. In fact, the higher concentration of 7.0 mg/kg did not show these adaptation mechanisms, as a significant decrease of GSHt took place, possibly as a result of MTX conjugation with GSH. The biological age of the mice is crucial to the antioxidant defense potential and to their detoxification mechanisms. In experiment 2, hepatic energetic impairments in MTX-treated animals occurred, excepting in 7.0 mg/kg MTX-treated pediatric mice. Cardiac histology showed that both populations of experiment 2 had inflammatory activity, cellular degeneration, with cellular edema and vacuolization, as well as some sporadic zones of necrosis, but with a lower degree in pediatric mice.

Altogether, these results suggest that pediatric mice might be more protected from damage induced by MTX than adult mice. However, time is a major handicap to appraise the mechanisms involved in the cardiotoxicity of MTX, since the animals may have time-dependent compensatory mechanisms. Thus, further investigation is needed to elucidate and to prove the parameters altered in both populations.

KEYWORDS: mitoxantrone; cardiotoxicity; cancer; children.

RESUMO

A terapia anticancerígena tem evoluído com a introdução de novos fármacos que aumentam a esperança média de vida. Contudo, o aumento de eficácia leva a inúmeros efeitos secundários e dano, especialmente para tecidos não cancerígenos. Desta forma, a sobrevivência ou qualidade de vida dos pacientes tratados estão comprometidas. A mitoxantrona (MTX) é um anticancerígeno largamente utilizado e um dos seus efeitos adversos principais é a toxicidade cardíaca que pode afectar até 18% dos pacientes tratados. Um dos mais preocupantes efeitos secundários cardíaco causado pela MTX é a falha cardíaca, que pode ser detectada apenas anos após a cessação da terapia anticancerígena. Embora a MTX tenha um perfil de toxicidade clínica semelhante à doxorrubicina, os seus mecanismos de cardiotoxicidade são diferentes, sendo largamente desconhecidos no caso da MTX. A MTX é igualmente utilizada em cancro pediátrico e em crianças com esclerose múltipla. Crianças que sobrevivem ao cancro têm potencialmente uma longa esperança média de vida após o tratamento, contudo pouco é conhecido acerca dos riscos desta população desenvolver toxicidade cardíaca após o tratamento com a MTX.

Assim, o presente estudo tem como principal objectivo avaliar parâmetros e factores de risco associados à toxicidade cardíaca provocada pela MTX, assim como a toxicidade causada por esta ao fígado e rins, em ratinhos CD-1 machos jovens (3 semanas) e adultos (8-10 semanas). Doses cumulativas totais de 4.5 mg/kg e 9.0 mg/kg de MTX foram administradas como resultado de 6 injecções intraperitoneais (2 por semana) na experiência 1. Doses cumulativas totais de 7.0 mg/kg e 9.0 mg/kg MTX foram utilizadas na experiência 2. O protocolo de administrações múltiplas foi utilizado de modo a mimetizar a terapia humana com MTX, que inclui a múltipla administração de MTX em diferentes ciclos. Após a administração da MTX, os animais foram mantidos, antes do sacrifício, por um período livre de fármaco de modo a permitir o desenvolvimento de toxicidade cumulativa. Na experiência 1, os animais que sobreviveram foram sacrificados no dia 20 após a última administração de MTX; na experiência 2, os animais tratados com 9.0 mg/kg MTX foram sacrificados um dia após a última administração, enquanto os animais tratados com 7.0 mg/kg MTX foram sacrificados 14 dias após a última administração de MTX. O consumo de comida e de água e o peso dos animais foram registados ao longo das experiências. Após o sacrifício, os seguintes parâmetros foram avaliados: glutationa total (GSHt), glutationa oxidada (GSSG), peroxidação lipídica e trifosfato da adenosina (ATP) no coração, fígado e rins; apoptose e exame histopatológico do coração; e, por último, os níveis plasmáticos de aminotransferase do aspartato (AST), aminotransferase da alanina (ALT), quinase da creatina total (total-CK) e isoenzima MB da quinase da creatina (CK-MB).

Nos estudos desenvolvidos na presente dissertação, a população pediátrica revelou ser mais resistente à toxicidade induzida pela MTX visto que, na experiência 1, alguns desses animais sobreviveram com a dose cumulativa total de 9.0 mg/kg de MTX. Em ambas as experiências, o peso médio animal dos ratinhos pediátricos quase não variou ao longo do tempo, em oposição ao que aconteceu com os ratinhos adultos. Elevações da AST nos animais tratados com 9.0 mg/kg na experiência 1 podem indiciar dano cardíaco, visto que os níveis de ALT não sofreram qualquer alteração. Os decréscimos na AST e ALT nos adultos tratados com a dose cumulativa de 4.5 mg/kg MTX podem dever-se à perda substancial de massa corporal ocorrida ou a outras mudanças metabólicas. Os níveis de CK-MB nos ratinhos pediátricos tratados com a dose cumulativa de 7.0 mg/kg MTX estavam aumentados, ao passo que na dose cumulativa de 9.0 mg/kg estavam diminuídos, o que revela que a concentração plasmática deste parâmetro é muito sensível a alterações fisiológicas. Não houve diferenças significativas nos níveis de peroxidação lipídica em nenhum grupo ou órgão, excepto no fígado dos ratinhos pediátricos tratados com 9.0 mg/kg MTX na experiência 2. Nesse órgão e grupo, a peroxidação lipídica diminui. Na experiência 1, a avaliação do conteúdo de glutationa no coração mostrou um aumento do rácio de GSH/GSSG nos ratinhos pediátricos tratados com 9.0 mg/kg, indiciando que estes podem ter sofrido uma adaptação nos níveis das suas defesas antioxidantes ao longo do tempo. De facto, quando a população foi sacrificada 24h após a última administração de MTX na mesma dose cumulativa, nenhuma alteração foi observada. Em oposição, os adultos tratados com 9.0 mg/kg MTX na experiência 2 apresentaram níveis maiores de GSSG. No coração dos animais tratados com 7.0 mg/kg MTX, nenhuma alteração significativa foi encontrada nos valores de glutationa nos ratinhos adultos, enquanto a população pediátrica teve níveis aumentados de GSHt. Neste grupo, os níveis de GSSG tiveram um ligeiro, mas não significativo aumento, ao passo que nenhuma alteração no rácio de GSH/GSSG foi observada. Assim, os mecanismos de adaptação das vias que envolvem a GSH não ocorreram nesta concentração (e tempo de sacrifício) nos ratinhos pediátricos. Os fígados dos ratinhos pediátricos tratados com 9.0 mg/kg MTX mostraram uma diminuição do rácio de GSH/GSSH na experiência 1, portanto o coração e fígado apresentam respostas diferentes em relação à MTX. Contudo e ainda no que diz respeito ao fígado, os adultos tratados com 4.5 mg/kg MTX tiveram um aumento significativo do rácio de GSH/GSSG, talvez devido a uma activação de mecanismos compensatórios que levam ao aumento de síntese da GSH em resultado do dano hepático provocado pela MTX. De facto, os animais da concentração cumulativa de 7.0 mg/kg não mostraram esses mecanismos de adaptação, havendo lugar a um decréscimo significativo de GSHt, possivelmente como resultado da conjugação da MTX com GSH. Estes resultados relacionados com fenómenos de stress

oxidativo sugerem que a idade biológica dos ratinhos influencia as defesas antioxidantes e os mecanismos de destoxificação. Na experiência 2, no fígado, ocorreu uma diminuição dos níveis de ATP nos animais tratados, excepto nos que foram tratados com a dose de 7.0 mg/kg. Relativamente à histologia cardíaca, ambas as populações da experiência 2 tratadas com MTX mostraram actividade inflamatória, degenerescência celular com edema celular e vacuolização, assim como zonas esporádicas de necrose. Estes danos ocorreram com menor grau de severidade nos ratinhos pediátricos.

Em resumo, estes resultados sugerem que os ratinhos pediátricos poderão estar mais protegidos do dano provocado pela MTX do que os ratinhos adultos. Contudo, o factor tempo é o maior entrave na avaliação dos mecanismos envolvidos na cardiotoxicidade da MTX, visto que os animais podem apresentar mecanismos compensatórios ao longo do tempo. Assim, investigação adicional é necessária para elucidar e comprovar os parâmetros observados em ambas as populações.

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ABBREVIATIONS

ATP – adenosine 5′-triphosphate

BHT – butylated hydroxytoluene

BNP – B-type (or brain) natriuretic peptide

BSA – body surface area

CHF – congestive heart failure

CK – creatine kinase

CK-MB – isoenzyme MB of creatine kinase

cTnI – troponin I

cTnT – troponin T

DNA – deoxyribonucleic acid

DNR – daunorubicin

DNROL – daunorubicinol

DOX – doxorubicin

DOXOL – doxorubicinol

ECG – electrocardiogram

EPIOL – epirubicinol

FDA – U.S Food and Drug Administration

γ-GCS – γ-glutamylcysteinesynthase

GSH – reduced glutathione

GSHt – total glutathione

GSSG – oxidized glutathione

$HO^{\bullet}$ – hydroxyl radical

H_2O_2 – hydrogen peroxide

HPLC – high-performance liquid-chromatography

i.p. – intraperitoneal

i.v. – intravenous

h – hour

HER2 – human epidermal growth factor receptor 2

LVEF – left ventricular ejection fraction

MDA – malondialdehyde

min – minute

MRP – multidrug resistance-associated protein

MTX – mitoxantrone

NADH – nicotinamide adenine dinucleotide

NADPH – nicotinamide adenine dinucleotide phosphate

$^{\bullet}NO$ – nitric oxide

$O_2^{\bullet -}$ – superoxide anion radical

$ONOO^-$ – peroxynitrite

PBS – phosphate buffered saline

RNA – ribonucleic acid

RNS – reactive nitrogen species

ROS – reactive oxygen species

SD – standard deviation

TBA – thiobarbituric acid

TCA – trichloroacetic acid

1. INTRODUCTION

1.1. Cancer nowadays

Presently, cancer is one of the most concerning health issues in the world. There were over 3.45 million new cases of cancer (excluding non-melanoma skin cancers) and 1.75 million deaths in Europe in 2012, with breast and prostate cancers counting for more than 800.000 new cases (Ferlay et al., 2013). Nowadays, in Portugal, cancer is the second major cause of death, after cardiovascular diseases (Adão et al., 2013). Even so, over the past years, the success of oncologic therapy has been impressive, improving significantly the survival and life-spam of cancer patients. As the treatment becomes more effective, more patients survive to cancer and the long-term side effects become an increasingly important issue. The ideal anticancer drug with maximal activity and neglectful toxicity to non-cancer cells is not yet found. Thus, anticancer treatment side effects still impair the integrity of healthy organs, specially the heart (Scully and Lipshultz, 2010, Adão et al., 2013). In fact, drugs such anthracyclines, anthracenediones, alkylating agents, antimetabolites, and antibodies are extensively implemented in cancer treatment and are well known for their cardiotoxicity (Ewer and Suter, 2010, Scully and Lipshultz, 2010).

1.2. Anticancer drugs and their cardiotoxicity

Several drugs have shown toxicity towards the heart. Anthracyclines and mitoxantrone (MTX) have been widely described as cardiotoxic (Seiter, 2005, Menna et al., 2008b, Adão et al., 2013, Todaro et al., 2013). Others agents such as the alkylating agents cyclophosphamide, ifosfamide and cisplatin; paclitaxel, 5-fluorouracil, amsacrine, tyrosine kinase inhibitors, and many others have been associated to cardiotoxicity (Costa et al., 2013b). In the introduction of this thesis, anthracyclines and MTX will be addressed for their cardiotoxicity in independent sections: anthracyclines for the wide literature available on the matter and MTX as it is the molecule of study in the present dissertation.

Anthracyclines and similar drugs (like MTX) that cause predominantly irreversible damage and impair myocardial contraction are designated as type I chemotherapy-related cardiac dysfunction agents (Table 1) (Ewer and Ewer, 2010). Although the cardiac damage is dependent on the cumulative dose, irreversible damage occurs in every administration of the drug and a second administration adds burden to an organ already injured. Doxorubicin

(DOX), epirubicin (EPI), and daunorubicin (DNR) are among the most commonly used antineoplastic agents in the treatment of a high variety of solid tumors and blood cancers (Ewer and Ewer, 2010, Ewer and Suter, 2010), as discussed below.

Agents that do not induce irreversible cardiac damage as a dominant characteristic are classified as type II agents. The ability of the cells to potentially remain viable after exposure to such agents will depend of several yet unknown factors (Ewer and Ewer, 2010, Ewer and Suter, 2010). Type II drugs cause reversible damage and do not have cumulative dose-dependent toxicity. Thus, these drugs may be administered for years without concern to their cumulative dose toxicity potential, and may be reintroduced in the therapy after cardiac recovery when heart damage occurred in the past (Ewer and Ewer, 2010). However, the question of synergy between the two types of agents is a matter of concern, when combining old-fashioned cytotoxics / cytostatics with the new generation of “targeted” drugs, such as trastuzumab and tyrosine kinase inhibitors (Menna et al., 2008a). Cardiotoxicity induced by anthracyclines and MTX can occur in low cumulative doses, if they are administered in combination with other agents, like trastuzumab, taxanes or cyclooxygenase-2 inhibitors (Minotti et al., 2004). In fact, cardiotoxicity is a problem when antibodies against human epidermal growth factor receptor 2 (HER2 or ErbB2) or tyrosine kinase inhibitors are utilized in clinical practice, especially when administrated with classical anticancer drugs. The most common and well known example for “targeted” drugs-induced cardiotoxicity is the above mentioned trastuzumab, a humanized monoclonal antibody directed against HER2, a receptor that is overexpressed in 15-25% of human breast cancers (Menna et al., 2012, Adão et al., 2013). HER2 is a transmembrane protein with a cytoplasmic tyrosine kinase domain. *In vivo*, neuregulin binding causes dimerization of HER2 with HER4 and the autophosphorylation of the dimer increases tyrosine kinase activity and activation of particular downstream signals (Menna et al., 2012). In cardiomyocytes, the neuregulin pathway is involved in several important mechanisms and activates survival signaling factors, namely growth factor receptor-bound protein 2 (Grb2), ras, Raf, mitogen-activated protein kinase (MAPK), phosphatidylinositide 3-kinase (PI3K) and protein kinase B (PKB, also known as Akt). These survival pathways modulate gene expression, cell growth / survival, glucose uptake, and the protein turnover of sarcomeres (Menna et al., 2008b).

Table 1 - Classification of agents regarding their cardiac side effects.

	Type I (myocardial damage)	Type II (myocardial dysfunction)
Characteristic agent	Doxorubicin	Trastuzumab (monoclonal antibody)
Findings on biopsy	Vacuoles, sarcomere / myofibrillar disruption, apoptosis and necrosis	Benign ultrastructure appearance
Mechanisms of cardiotoxicity	ROS formation, oxidative stress/damage, energy collapse, and calcium and iron dysregulation	Block HER2 signaling pathway, elimination of HER2/HER4-related survival factors (gene expression, growth, glucose uptake, sarcomere turnover)
Cumulative dose relationship	Yes	No
Reversibility	No, damage appears to be permanent and irreversible; may stabilize, but subclinical damage persists	Yes, high probability of recovery after therapy interruption (up to 79%)
Cardiovascular effects	May result in incurable heart failure and death	Low probability of cardiovascular mortality
Other agents	Daunorubicin, epirubicin, idarubicin (anthracyclines), mitoxantrone (anthracenedione), cyclophosphamide (oxazophorine alkylating agent)	Sunitinib, lapatinib (tyrosine kinase inhibitors)

Adapted from Ewer & Ewer (2010), Ewer & Lippman (2005), Menna *et al* (2008b), and Todaro *et al* (2013).

The binding of trastuzumab to the extracellular domain of HER2 leads to inhibition of the neuregulin signaling pathway in cancer and also in the heart (Adão et al., 2013). The factors associated to HER2 play a crucial function in the survival and development of cardiomyocytes. The binding of trastuzumab to HER2 impairs the mitochondrial integrity through the family of BCL-X, leading to depletion of adenosine triphosphate (ATP) and subsequent contractile dysfunction in cardiomyocytes (Adão et al., 2013). When subjected to cardiac stress, an increase of HER2 expression and activation of the HER2-HER4 signaling by neuregulin occurs, as a protective mechanism (Adão et al., 2013).

In fact, if cardiomyocytes are exposed to type I agents, the cardiac HER2 activation becomes important by preventing energy collapse, sarcomere disarray, apoptosis or necrosis. Blocking HER2 with trastuzumab will exacerbate anthracycline-induced cardiotoxicity, since these survival factors and repair mechanisms will be reduced in the heart (Figure 1) (Menna et al., 2008b). This combined therapy increases reactive oxygen species (ROS) and reduces antioxidant defenses, leading to oxidative stress, cardiac dysfunction, and consequently overexpression of angiotensin II (Adão et al., 2013). The increased levels of angiotensin II inhibit the action of neuregulin, preventing it from binding to

HER receptors and the activation of anti-apoptotic signaling pathways (Adão et al., 2013). In addition, angiotensin II activates the nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase via angiotensin type 1 (AT1) receptor and protein kinase C. Moreover, NADPH oxidase is a potent enzyme in producing superoxide radical anion ($O_2^{\bullet -}$), and AT1 signaling is related with the activation of apoptosis signaling kinase 1 (ASK1), promoting cell death and cardiac dysfunction (Adão et al., 2013).

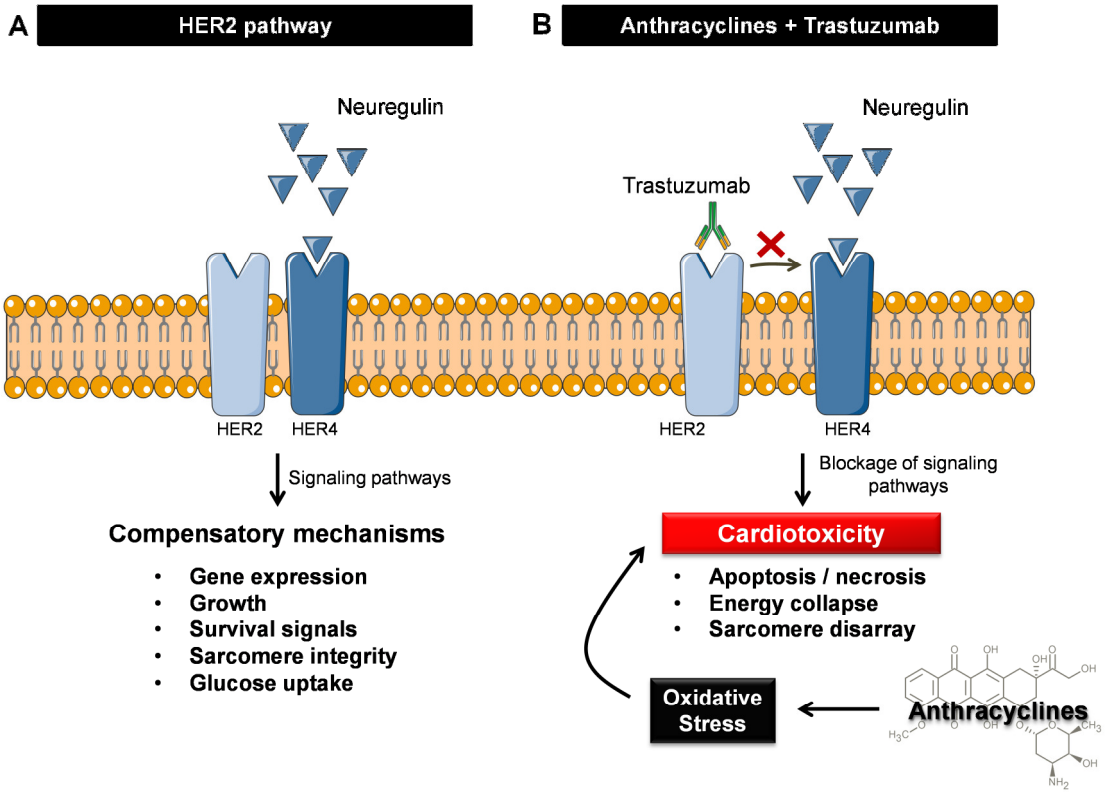

Figure 1 - (A) HER2 pathways and their protective effect in the heart. Heterodimerization of HER2-HER4 due to HER2 binding of neuregulin activates life-oriented signals and survival factors that minimize cardiotoxicity when the heart is exposed to anthracyclines; (B) Anthracyclines induce cardiotoxicity via oxidative stress mechanisms. Trastuzumab burdens cardiotoxicity by blocking the heterodimerization of HER2-HER4 and therefore the downstream signaling that is responsible for compensatory mechanisms.

1.3. Risk factors for cardiotoxicity in anticancer therapy with type I agents

There are considerable risk factors that contribute for cardiotoxicity when therapy with type I agents is required (Table 2). When the effect of anticancer agents is analyzed in perspective and in order to diagnose the cardiovascular effects resulting of therapy, several variables should be taken into account. Moreover, several of the variables that increase the

incidence of cardiotoxicity, change with disease progression (Scully and Lipshultz, 2010). Damage depends of several pharmacological factors as the total cumulative dose (being the principal risk factor), type of drug, route and rate of administration, dosing schedule, concomitant pharmacological therapy, and irradiation (Scully and Lipshultz, 2010, Adão et al., 2013). Other factors linked to the patient's characteristics such as age, sex, presence of other co-morbidities or genetic factors and inter-patient variability are also important for cardiotoxic events (Lipshultz et al., 2008, Scully and Lipshultz, 2010, Adão et al., 2013). In addition, many toxic processes can be exacerbated directly by the tumor, namely by causing local inflammation close to the heart, or act as an arrythmogenic focus (Ewer and Ewer, 2010).

Table 2 - Risk factors for anthracycline and MTX-induced cardiotoxicity.

Risk factors	Increased risk
Total cumulative dose [a]	Most important predictor of cardiac dysfunction Exceeding: Daunorubicin 550 - 800 mg/m^2 Doxorubicin 400 - 550 mg/m^2 Epirubicin 900 - 1000 mg/m^2 Idarubicin 150 - 225 mg/m^2 Mitoxantrone 140 mg/m^2
Age	Pediatric and elderly are more prone to greater cardiotoxicity (in the same cumulative dose)
Gender	Feminine patients are more prone to greater cardiotoxicity (in the same cumulative dose)
Mode of administration	Rapid intravenous injection
Combination chemotherapy (e.g. cyclophosphamide, trastuzumab, and paclitaxel)	Concomitant exposure to cardiotoxic agents may predispose to cardiotoxicity
Prior/concomitant mediastinal radiotherapy	Evidence of enhanced cardiotoxicity (additive or synergistic)
Previous cardiac disease (coronary, valvular or myocardial) and hypertension	Higher risk of early clinical cardiotoxicity
Electrolyte disturbances	Hypocalcaemia, hypomagnesaemia

Adapted from Adam & Lipshultz (2005), Adão *et al.* (2013), Costa *et al.* (2013), Menna *et al.* (2008b) and Singal & Iliskovic (1998).

[a] Total cumulative dose should be seen as "lifetime" cumulative dose, i.e. should be understood as the arithmetic sum of all individual doses administered to a patient, even with years or months between cycles (Menna et al., 2008a).

One of the main aspects that contribute to the cardiotoxicity of type I agents is the cumulative dose administered. Body-surface area (BSA) is commonly used for the calculation of the dose of anticancer drugs to be administered and is measured in square

meters (m^2). The calculation can be done using the following expression, which relates height and weight from the patient:

$$\text{Surface area} = \sqrt{\frac{\text{Height(cm)} \times \text{weigh(kg)}}{3600}}$$

Figure 2 - Mathematical formula to calculate body-surface area (BSA).

There is a dose relationship between cardiac toxicity and the cumulative administered dose of anthracyclines and MTX (Figure 3 and Table 2). Although there is little concern about cardiotoxicity at low cumulative doses, the cardiotoxicity relation curve grows rapidly as the cumulative dose increases. In a retrospective analysis, Von Hoff *et al.* reported that there is a continuous increasing risk with the increase of total cumulative dose of those anticancer agents, estimating the cumulative percentage of patients who developed congestive heart failure (CHF) at a cumulative dose of DOX of 400 mg/m^2 to be 3%, 7% at 550 mg/m^2, and 18% at 700 mg/m^2. Others authors discuss that Von Hoff study had some restraint and perhaps underestimated the incidence. In a study by Swain *et al.* the estimated cumulative percentage of patients with CHF associated with DOX reaches 5% of patients at a cumulative dose of 400 mg/m^2, rising to 26% at a dose of 550 mg/m^2, and 48% at a dose of 700 mg/m^2 (Figure 3) (Swain et al., 2003). The usual administered dose of DOX depends of the type of tumor, although the most common dose is 60-75 mg/m^2 every 3 weeks (Seiter, 2005). It should be noted that late asymptomatic cardiomyopathy can occur at any dose, even as low as 100 mg/m^2 (Menna et al., 2012).

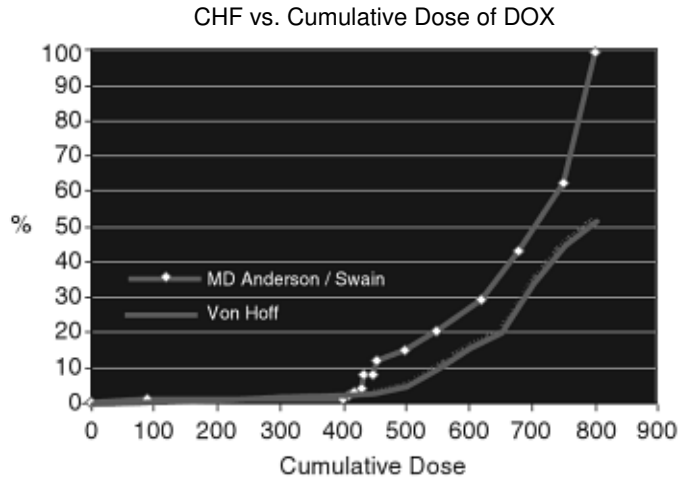

Figure 3 - Congestive heart failure (CHF) versus cumulative dose for doxorubicin, adapted from Ewer & Suter, 2010.

Age is also a crucial factor for the development of cardiotoxicity in type I anticancer drugs. The cardiac damage that occurs in childhood may appear early after cancer treatment, or, more frequently, it is diagnosed years later. In general, the younger the patient, the greater the susceptibility and risk for developing delayed cardiac damage, worsening when there are synergistic and additive factors as concomitant cardiotoxic therapies. In the literature, it is stated that DOX-induced cardiomyopathy impairs myocardial growth as the younger patients mature, leading to a possible development of CHF during early childhood (Adams and Lipshultz, 2005, Seiter, 2005). In a study from the Childhood Cancer Survivor Study on children diagnosed with cancer in the 70s and 80s, they observed that childhood survivors were at 15.1-fold higher rate of developmental CHF and 10.4-fold higher rate of developmental cardiovascular disease, compared to siblings (Oeffinger et al., 2006). This topic will be further addressed later on in this dissertation in an independent section.

The cardiotoxic effects of anthracyclines are also higher in women, black children and patients with trisomy 21, as well as in the presence of concomitant diabetes, obesity, kidney dysfunction, and others (Krischer et al., 1997) (Table 2). One possible explanation for the increased susceptibility to myocardial damage in females resulting from anthracycline therapy is the highest percentage of body fat compared to males. As anthracyclines are poorly absorbed in fat, cellular concentrations tend to be higher in non-adipose tissue in patients that receive doses calculated by BSA or weight (Krischer et al., 1997). Another possible reason may be related to sex differences in the expression of multidrug-resistance genes, which control cellular excretion of anthracyclines; the sex-related differences in the pharmacokinetics of anthracyclines demonstrate that females have a lower clearance rate (Krischer et al., 1997).

Treatment duration may also influence the risk of developing cardiotoxicity during or after oncologic therapy, since prolonged administration has been known to reduce the severity of cardiac damage (Hortobagyi et al., 1989). Other risk factors include the existence of previous cardiac disease at the beginning of the treatment or electrolyte disturbances, namely of calcium and magnesium (Adão et al., 2013).

1.4. Cardiotoxicity detection

Some guidelines exist for interruption of anticancer treatment when unacceptable toxicity is observed (Bovelli et al., 2010, Eschenhagen et al., 2011). Thus, it is vital to implement cardioprotective strategies and monitor cardiac function during therapy. It is possible to evaluate some toxic effects using cardiac imaging studies such as echocardiography to

identify myocardial dysfunction, or even endomyocardial biopsy for measuring the presence and extent of fibrosis (Schimmel et al., 2004). A decline of left ventricular ejection fraction (LVEF) higher than 10% associated with an absolute value of less than 50% is usually a clinical setting to stop the cardiotoxic treatment (Colombo and Cardinale, 2013). However, these detection methods have low sensitivity for detecting cardiac damage at an early stage, since no considerable change in ventricular systolic function occurs until a critical myocardial damage has been made. Furthermore, cardiotoxicity is commonly detected only after functional impairment has already occurred, therefore excluding its prevention. In fact, a normal LVEF does not exclude *per se* the possibility of occurrence of late cardiotoxicity (Colombo and Cardinale, 2013).

Simplest minimally invasive and low cost methods such plasma or serum cardiac markers have been investigated to monitor the anthracycline-induced cardiomyopathy, making them sensitive tools for detection of early damage, with high prognostic value (Colombo and Cardinale, 2013). A known marker for heart damage is the cardiac troponins I and T (cTnI and cTnT), which are elevated in the serum in case of myocyte injury, even when a subclinical alteration occurs with minor anthracyclines exposure (Franco et al., 2011, Colombo and Cardinale, 2013). Most of the troponins are present in the cardiac myocyte sarcomere, attached to actin and are released slowly, allowing the detection of acute damage, as well as ongoing injury (Lipshultz et al., 2008, Scully and Lipshultz, 2010). The monitoring of troponin levels is, thus, useful for prophylactic cardioprotective therapy before occurrence of irreversible damage. Another well-known cardiac marker for CHF is serum B-type (or brain) natriuretic peptide (BNP) hormone that is secreted by cardiomyocytes due to overload pressure in the heart, as well as left ventricular dysfunction. It is useful as early stress indicator before irreversible damage occurs (Schimmel et al., 2004, Ewer and Ewer, 2010, Scully and Lipshultz, 2010, Franco et al., 2011). High sensitivity serum C reactive protein (HsCRP) is also a cardiac marker and evidences generalized inflammation being a predictor of ischemic and non-ischemic cardiomyopathy (Lipshultz et al., 2008, Franco et al., 2011). Other conventional biomarkers used for assessing myotoxicity in humans and experimental animals for decades include aspartate aminotransferase (AST) and creatine kinase (CK) in the plasma, but they have some drawbacks regarding tissue specificity (Tonomura et al., 2012). The myocardial isoenzyme of CK, namely CK-MB, is a cardiospecific biomarker that represents up to 30% of total-CK in heart and is released when cardiomyocytes are injured (Adams et al., 1993, Lewandrowski et al., 2002, Horacek et al., 2007, O'Brien, 2008).

1.5. Anticancer drugs and their cardiotoxicity

1.5.1. Anthracyclines

Anthracyclines are considered a class of highly effective anticancer drugs with the widest spectrum of activity in human cancers. The skeleton of anthracyclines is composed of a tetracyclic ring with adjacent quinone-hydroquinone moieties, an aminosugar attached by a glycosidic bond to C-7, and a side chain with a carbonyl group at C-13 (Figure 4) (Menna et al., 2012). There is no safe dose of anthracyclines. During the last years, there have been several attempts to find new anthracyclines with better activity and less toxicity to cardiac tissue than DOX and DNR (Minotti et al., 2004, Salvatorelli et al., 2013). Thus, hundreds of analogs have arisen with small modifications that can be placed in the various available locations in the molecule, but few analogs reached the stage of clinical development and approval: the popular epirubicin and idarubicin as substitutes for DOX and DNR, respectively. Beyond these, only a few more anthracyclines reached clinical approval: among them, MTX, an anthracenedione, was used in attempt to overcome anthracyclines cardiotoxicity (Minotti et al., 2004).

The first anthracyclines, isolated from *Streptomyces peucetius*, were developed in the early 1950s: DOX and DNR (Minotti et al., 2004). This last molecule differs from DOX only by not having a hydroxyl group in the side chain (Figure 4), which contributes for the different spectrum activity of DOX and DNR. DOX is clinically and widely used in the treatment of breast cancer, childhood solid tumors, soft tissue sarcomas and lymphomas, while DNR exhibits activity in acute lymphoblastic or myeloblastic leukemia's (Minotti et al., 2004). Anthracyclines have cytostatic effects in tumor cells related to their multiple pharmacological mechanisms of action: intercalation in deoxyribonucleic acid (DNA), inhibition of topoisomerase II, and prevention of synthesis of nucleic acids [(DNA and ribonucleic acid (RNA)] and proteins (Ewer and Suter, 2010). These cytotoxic drugs are well known for their cardiotoxicity (Schimmel et al., 2004).

More than ever, cardiovascular diseases in cancer patients are a matter of concern for cardiologists and oncologists. The use of anticancer drugs, in particular cytotoxic agents, has raised several problems since they trigger multiple adverse effects in healthy tissues, namely the heart, with cardiotoxicity being the dose limiting factor in cancer treatment (Schimmel et al., 2004, Ewer and Ewer, 2010). The heart is an organ with limited potential for regeneration and, therefore, its damage is considered permanent and with long-term importance (Ewer and Ewer, 2010).

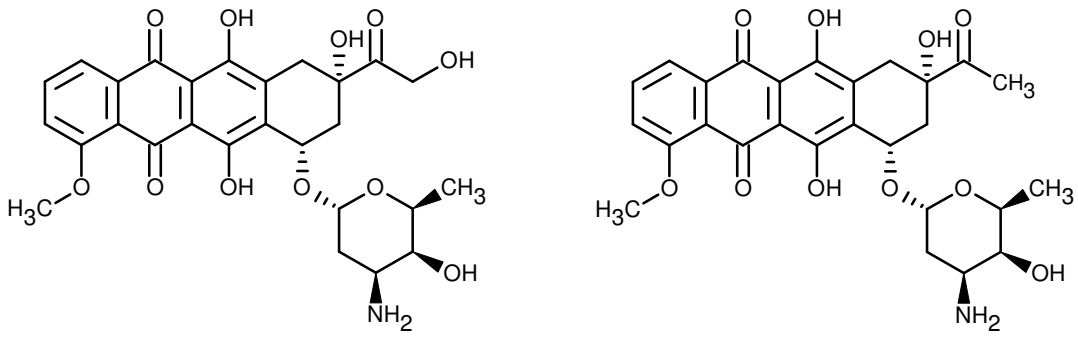

Figure 4 - Chemical structure of doxorubicin (DOX) and daunorubicin (DNR).

1.5.1.1. Cardiotoxicity of anthracyclines

The anthracycline-induced cardiotoxicity can be divided into two categories: acute and chronic. The acute-induced cardiotoxicity symptoms occur within a week after administration (<1%) (Chen et al., 2011), are dose-independent and are characterized by sudden alterations of ventricular repolarization, electrocardiographic alterations, ventricular and supraventricular arrhythmias, acute coronary syndrome, pericarditis, and myocarditis (Adão et al., 2013). The chronic induced cardiotoxicity is divided in early-onset when it occurs within 1 year after completing treatment and it is related to myocyte damage or death (<2.1%) (Krischer et al., 1997, Chen et al., 2011); and in late-onset, when it occurs after the first year and is related to depressed contractility and inappropriately thin left ventricular wall (5-50%) (Schimmel et al., 2004, Chen et al., 2011, Adão et al., 2013). The late chronic cardiotoxicity of anthracyclines is dose-dependent (Adão et al., 2013) and can be manifested years after the discontinuation of treatment (Ewer and Suter, 2010, Scully and Lipshultz, 2010). It is also important to refer that the late-onset form may remain asymptomatic for years (Colombo and Cardinale, 2013). In all these categories, electrophysiological changes, decrease of LVEF, reduced exercise ability, and CHF may occur and are dependent of the rate and schedule of therapy (Schimmel et al., 2004, Adams and Lipshultz, 2005, Ewer and Ewer, 2010, Scully and Lipshultz, 2010). Besides cardiomyocyte damage, the anthracyclines are also able to cause damage to endothelial cells (Octavia et al., 2012).

The clinical manifestations of late-onset toxicity often appear years after therapy cessation. Compensatory mechanisms are activated in the heart after injury, including

adrenergic nervous system, renin-angiotensin system, survival factors, and adaptive myocardial hypertrophy (Mann and Bristow, 2005, Lipshultz et al., 2008, Ewer and Suter, 2010). In fact, these systems are able to restore hemodynamic cardiovascular function to a normal homeostatic range and the side effects in patients may remain asymptomatic. Unfortunately, these compensatory mechanisms may become exhausted over time and the heart will begin to fail due to the sustained activation of these systems. When compensating mechanisms are overcome, patients may exhibit progressive tachycardia, fatigue, and difficulties in breathing, then undergoing from asymptomatic to symptomatic heart failure (Figure 5) (Mann and Bristow, 2005, Ewer and Suter, 2010).

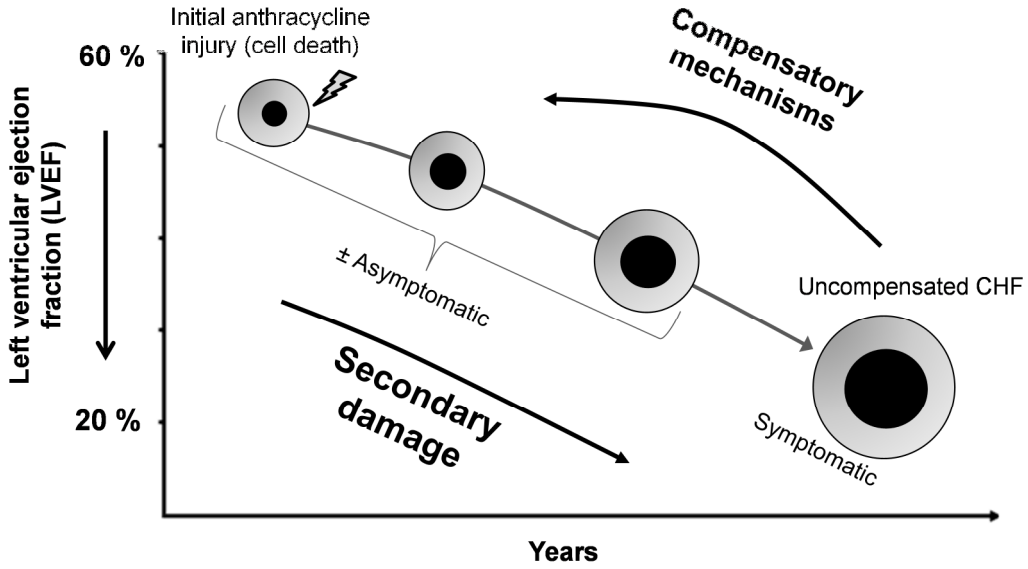

Figure 5 - Progression of the myocardial injury to congestive heart failure (CHF) caused by anthracyclines. Adapted from Mann & Bristow (2005) and Ewer & Suter (2010).

1.5.1.2. Anthracyclines and their mechanisms of cardiotoxicity

The underlying mechanisms of anthracyclines cardiotoxicity are complex and still being explored (Ewer and Suter, 2010). Currently, it is thought that anthracyclines may become cardiotoxic after one or two electron reductive activation via enzymes, or through the formation of anthracycline-iron complexes (Minotti et al., 2004, Lipshultz et al., 2008, Montaigne et al., 2012). The predominant mechanism elicited by DOX involves oxidative damage: the one electron reduction in the quinone moiety of DOX results in the formation of the semiquinone free radical. This semiquinone free radical forms a complex with iron (Fe^{2+}) and is regenerated to quinone again by reduction of molecular oxygen to $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2) (Minotti et al., 2004, Montaigne et al., 2012), molecules belonging to the

family of ROS. This cycle is supported by a number of NADPH-oxidoreductases: cytochrome P-450 or b5 reductases, mitochondrial nicotinamide adenine dinucleotide (NADH) dehydrogenase, xanthine dehydrogenase, and endothelial nitric oxide synthase (Minotti et al., 2004, Montaigne et al., 2012, Costa et al., 2013b). The superoxide anion radical may react with nitric oxide ($^{\bullet}NO$), resulting in peroxynitrite ($ONOO^{-}$), highly reactive nitrogen species (RNS) (Menna et al., 2012), and DOX semiquinone can react with H_2O_2 to yield hydroxyl radical ($HO^{\bullet}$) (Costa et al., 2013b). These redox active molecules are responsible for dysregulation of energy metabolism, damage of cardiac mitochondrial DNA and membrane integrity in these vital cells (Schimmel et al., 2004, Scully and Lipshultz, 2010, Menna et al., 2012). The ROS and RNS formed can also interact with the calcium release channels, by altering calcium stores in sarcoplasmic reticulum (Pai and Nahata, 2000). Cardiac mitochondria are main targets for anthracyclines cardiotoxicity, since they have high affinity to a mitochondrial lipid, cardiolipin, and the mitochondrial NADH dehydrogenase intervenes in the one electron reduction (Costa et al., 2013b).

During the redox cycle, the anthracycline semiquinone can also be a target for oxidation, losing the sugar moiety, and leading to the formation of anthracycline aglycones. These are more lipophilic molecules that easily intercalate in the mitochondrial membranes (Costa et al., 2013b). Cardiac mitochondria seem to be susceptible to aglycones, since they can cause mitochondrial dysfunction by modifying sulfhydryl groups and induce calcium-independent oxidation of mitochondrial NADPH, leading to $O_2^{\bullet-}$ production. Furthermore, the accumulation of the lipophilic aglycones in the inner mitochondrial membrane diverts electrons from the normal pathways, affecting the energy metabolism and cellular respiration (Costa et al., 2013b).

The two electron reduction of the carbonyl side chain of anthracyclines is characterized by a conversion of the anthracyclines into secondary alcohol metabolites, such as doxorubicinol (DOXOL) or daunorubicinol (DNROL) and this conversion is catalyzed by cytoplasmic cardiac aldo/keto or carbonyl reductases (Menna et al., 2008b, Costa et al., 2013b). These alcohol metabolites are less active in redox cycle than the original quinones but are more potent in dysregulating calcium and iron homeostasis (Figure 6) (Menna et al., 2008b, Ewer and Ewer, 2010, Scully and Lipshultz, 2010). The increased available “free” iron in cells promotes Fenton reactions and consequently further oxidative stress (Minotti et al., 2004).

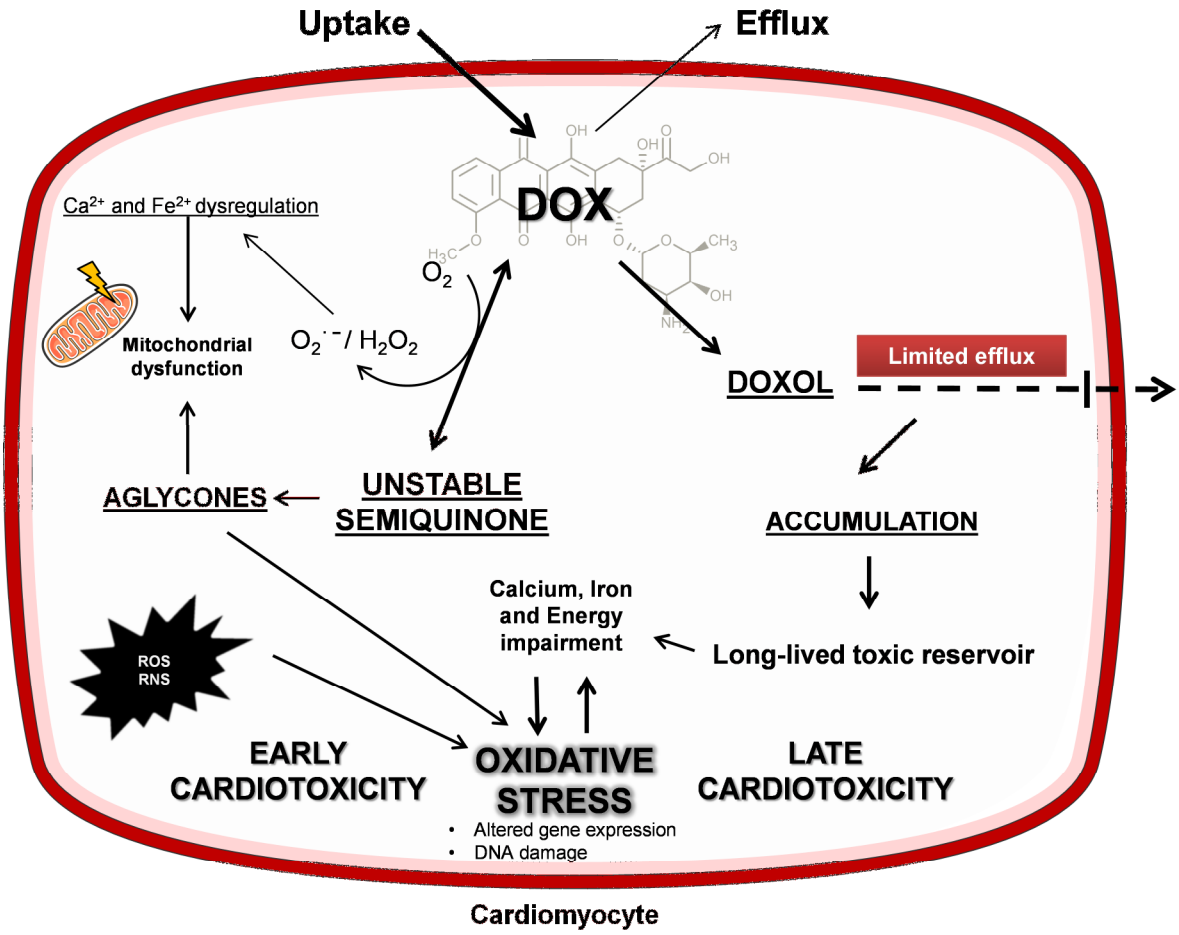

Figure 6 - Anthracyclines, such as doxorubicin (DOX), enter easily in cardiomyocytes through passive diffusion and become cardiotoxic after one- or two-electron reductive activation. The cardiotoxicity of anthracyclines is multifactorial. The cardiac damage caused by oxidative stress has probably two phases: early cardiotoxicity characterized by formation of semiquinones (one electron reduction) or aglycones that trigger an initial high oxidative stress burst and the late/chronic cardiotoxicity characterized by the less redox active but highly toxic secondary alcohols (two electron reduction), forming doxorubicinol (DOXOL) which forms a long-lived toxic reservoir due to its high hydrophilicity. See text for details.

The secondary alcohols have increased polarity and then remain more time with the cells and, therefore, their cardiac elimination is reduced when compared to anthracyclines. Hence, this cardiac accumulation in the heart forms a long-lived anthracycline reservoir, which may explain the reason why anthracyclines cause chronic cardiac toxicity (Figure 6) (Menna et al., 2008b, Menna et al., 2012). Moreover, DOXOL and DNROL are 30-40 times more potent than DOX and DNR in inactivating calcium-handling ATPases, ion channels or membrane ion exchangers (Menna et al., 2012). These alcohol metabolites are known to interact with thiol groups on proteins, leading to cell damage (Octavia et al., 2012). These alterations result in ROS that are responsible for lipid peroxidation and DNA, RNA, and protein synthesis damage, with alterations in calcium transport (e.g. sarco/endoplasmic reticulum Ca^{2+}-ATPase, SERCA) and reduced glutathione reductase gene expression. These changes result in tissue injury, cell death, and impaired cardiac contraction (Schimmel et al., 2004, Lipshultz et al., 2008, Menna et al., 2008b, Costa et al., 2013b). Apart from its limited

regenerative capacity, cardiomyocytes present a high susceptibility to oxidative damage due to their limited biochemical antioxidant reserves, namely ROS-detoxifying enzymes such as catalase and glutathione peroxidase (Pai and Nahata, 2000, Schimmel et al., 2004). The oxidative stress hypothesis for the anthracycline-induced cardiotoxicity was also demonstrated through the use of transgenic mice with overexpression of antioxidant defense systems. In fact, high resistance to DOX-induced cardiac lipid peroxidation was observed in transgenic overexpressing catalase mice (Kang et al., 1996) and the detoxification by scavenging superoxide radicals produced by DOX occurred in transgenic overexpressing mitochondrial manganese-dependent superoxide dismutase (Yen et al., 1996).

Anthracyclines accumulate in the heart at concentrations 10- to 500-times higher than their extracellular concentrations. Moreover, cardiolipin, a polyunsaturated fatty acid-rich phospholipid found in high concentrations in the inner mitochondrial membrane, has high affinity for anthracyclines, promoting high intramitochondrial levels of these drugs (Lipshultz et al., 2008). Therefore, in the mitochondria, the respiratory chain is inhibited when anthracyclines bind to cardiolipin or interact with mitochondrial DNA, favoring energy metabolism impairment and enhancing oxidative stress. The higher levels of cardiolipin within the mitochondria, the greater the susceptibility of cardiac cells damage by anthracyclines (Lipshultz et al., 2008, Scully and Lipshultz, 2010, Franco et al., 2011). Anthracyclines decrease ATP production by disrupting the cardiac specific gene expression of critical enzymes involved in energetics, as well as by disrupting structural gene products (cardiac troponins, myosin light chains, and CK) (Lipshultz et al., 2008). Decreases in ATP levels can be also due to the activation of apoptotic signaling and calcium-dependent proteases, which consume ATP and compromise protein reparation; in general, fix and replace damaged proteins represents a high energy expenditure (Octavia et al., 2012). The decrease in protein expression and the degradation of myofilaments lead to a negative balance of cardiac structural sarcoplasmic proteins (Dillenburg et al., 2013), such as titin, a very large myofilament protein, which aids the sarcomere return to the resting state during diastole as well as it regulates the initiating contraction during systole; and dystrophin, another large protein of the sarcomere, which acts as an anchor of the extracellular matrix to the cytoskeleton via actin (Chen et al., 2011). This cardiac contractility impairment can also be due to the direct inhibition and/or reduced expression of the calcium-ATPase that sequesters calcium in the sarcoplasmic reticulum (Lipshultz et al., 2008, Chen et al., 2011, Montaigne et al., 2012). Decreased energy levels impair the ability of cardiomyocytes to contract correctly, leading, in some cases, to cell death (Lipshultz et al., 2008).

Complementary mechanisms of anthracycline cardiotoxicity involve cardiac cells death by apoptosis or necrosis (Adão et al., 2013). It is usually accepted that the apoptosis signaling is activated by the oxidative stress caused by anthracyclines (Octavia et al., 2012).

Anthracycline-induced apoptosis in the heart appears to be linked to the mitochondrial pathways, requiring Bax, cytochrome *c* and caspase-3 activation (Figure 7) (Montaigne et al., 2012). Anthracycline increases mitochondrial oxidative stress and disrupts calcium levels, altering membrane permeability and its potential, and resulting in opening of the transition pore (mPTP), which is associated with the release of apoptotic factors such as cytochrome *c* from mitochondria to cytosol. In the cytosol, cytochrome *c* forms a complex with the adaptor protein apoptosis protease activator protein-1 (Apaf-1) and procaspase-9, named apoptosome, which activates caspase-9 (Montaigne et al., 2012). Anthracyclines also induce apoptosis by activation of p38 mitogen-activated protein kinases (MAPK), stress-activated protein kinase (SAPK), and c-Jun N-terminal kinases (JNK) through oxidative stress mechanisms (ROS and RNS) (Minotti et al., 2004, Montaigne et al., 2012), as well as by disrupting / down regulating the expression and activity of the transcription factor GATA-4 (Montaigne et al., 2012). GATA-4 is a member of the zinc finger transcriptional factor family and it has an important role in regulating differentiation, sarcomere synthesis, and survival signaling, and also in promoting an anti-apoptotic response in the heart (Montaigne et al., 2012). Anthracyclines-induced apoptosis of cardiac cells is prevented by several survival factors, namely by phosphoinositide kinase (PI3K) / Akt and neuregulin/HER2 activation (Minotti et al., 2004, Montaigne et al., 2012).

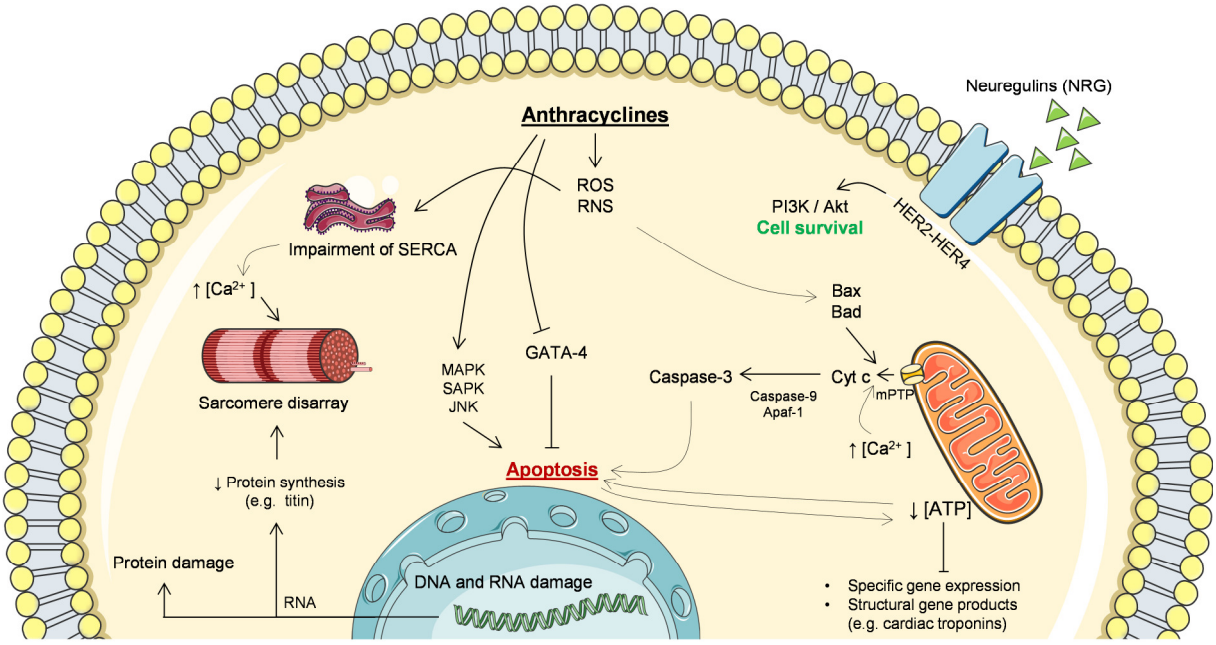

Figure 7 - Signaling pathways involved in anthracycline-induced toxicity. The principal mechanism of anthracycline damage is via the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to lipid peroxidation and membrane damage. In mitochondria, ROS and calcium overload lead to the release of cytochrome *c*, which activates caspases and leads to apoptosis. Other mechanisms include damage to nuclear DNA, disturbance of energetic metabolism, disruption of sarcomere, and suppression of transcription factors, namely GATA-4 that regulates cell survival.

Toxicity events may be related to many other factors beyond the ones mentioned above, such as induction of •NO synthase (Octavia et al., 2012). Additionally, anthracyclines inhibit regenerative pathways such as the HER1 pathway (Dillenburg et al., 2013). No matter the mechanisms, clinically the loss of myocytes causes thinning of the myocardium, and increases wall stress. As the remaining myocardial cells have defective regenerative and adaptive pathways, the adaptive responses to increase wall stress seem incomplete (Dillenburg et al., 2013). This leads to the progression of reduced contractility, cardiac remodeling and progressive ventricular fibrosis. Some of these mechanisms are depicted in Figure 7.

1.5.2. Mitoxantrone

Mitoxantrone (MTX), 1,4-dihydroxy-5,8-bis[(2-[(2-hydroxyethyl)amino]ethyl)amino]-9,10-anthracenedione, was semi-synthesized in 1979. It is a drug structurally related to the anthracyclines with a broad spectrum antitumor activity. It is commonly used in breast and prostate cancer, acute leukemia, and lymphomas. MTX was aimed to maintain or improve the anthracyclines antitumor activity and reduce their cardiotoxic side effects (Fox, 2004, Scully and Lipshultz, 2010, Ferlay et al., 2013). The main alteration of MTX comparing with anthracyclines was the replacement of the amino sugar on the anthracyclines for amino-containing groups (Figure 8) (Pratt et al., 1986). In 2000, MTX was approved by the U.S Food and Drug Administration (FDA) as an immunomodulation agent for reducing neurological disability of worsening relapsing-remitting multiple sclerosis, an autoimmune disease characterized by the progressive destruction of the myelin sheath surrounding axons in the central nervous system (CNS) (Avasarala et al., 2003, Seiter, 2005).

Different mechanisms have been attributed to the pharmacological actions of MTX based *in vitro* studies, which do not always correlate with the *in vivo* data. The main pharmacological features of MTX include intercalation in DNA, formation of crosslinks and strand breaks, and electrostatic interactions with the phosphate group of DNA by the basic side chains containing amino group of MTX. It was also shown that MTX preferably binds to polynucleotides containing guanosine-cytosine (Alberts et al., 1985). *In vitro*, MTX is a very effective inhibitor of DNA replication and affects various stages of the cell cycle, particularly during the mid-to-late-G1 and mid-to-late-G2, blocking the cell's entrance to mitosis. It also inhibits DNA-dependent RNA synthesis and topoisomerase II, an enzyme responsible for uncoil and repair of damaged DNA (Ehninger et al., 1990, Fox, 2004). The cytotoxic activity of MTX involves killing of both proliferating and non-proliferating cells and, thus, it has a non-cell-cycle-specific mechanism of action. Fundamentally, it leads to apoptosis (Fox, 2004).

Others authors demonstrated that the cytotoxic effect of MTX depends on the cytochrome P-450-mediated metabolism of the drug, since the inhibition of this group of enzymes leads to a complete loss of the drug-dependent inhibition of cell growth (Mewes et al., 1993). MTX in high concentrations also inhibits the biosynthesis of prostaglandins namely prostaglandin E2 and the release of calcium: prostaglandins have important roles in the metastization of tumors and hypercalcaemia in cancers (Novak and Kharasch, 1985).

Although MTX was first developed with the aim to replace anthracyclines' cardiotoxicity, it has been associated with impairment of cardiac function (Pai and Nahata, 2000, Scully and Lipshultz, 2010).

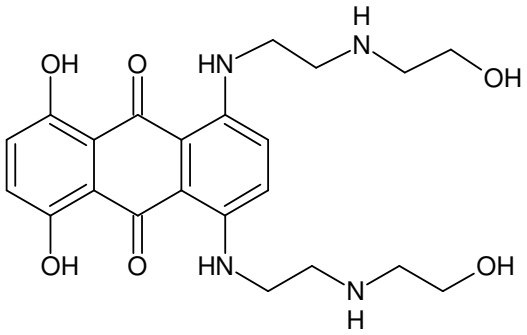

Figure 8 - Chemical structure of mitoxantrone (MTX).

Reporting to immunomodulation, MTX has immunosuppressive activity with mechanisms associated with suppression of T cells, B cells, and macrophages proliferation, reduction of secretion of pro-inflammatory cytokines IFN-γ, TNF-α, and IL-2 and impairment of antigen presentation (Fox, 2004, Scott and Figgitt, 2004). MTX is also able to kill normal cells responsible for deleterious autoimmune function (Fox, 2004).

1.5.2.1. Pharmacokinetics

MTX is poorly absorbed orally, so it is administered intravenously (i.v.). Although the dose of MTX ranges between 5-12 mg/m^2, the administration schedule can vary according to the disease and response to therapy, ranging from 4-6 weeks for the treatment of acute myeloid leukemia, 3 weeks for the treatment of prostate cancer, and 3 months for multiple sclerosis (Table 3) (Fox, 2004, Scott and Figgitt, 2004).

The pharmacokinetic curve that relates the plasma concentration of MTX with time has a tri-exponential equation, characteristic of a three-compartment open pharmacokinetic model: an initial α half-life of 4.1 to 10.7 min, corresponding to the moment when MTX rapidly leaves

the plasma and binds to the endothelial surface; an intermediate β half-life of 0.3 to 3.1 h, corresponding to the distribution phase; and finally a long terminal γ half-life, approximately 8.9 to 9 days. However, some authors reported longer times of elimination, going up to 12 days. This prolonged elimination may be associated with concomitant chemotherapy, that alters the elimination rate of MTX or even with the different sensitivities of the methods used, limiting the "true" terminal half-life (Batra et al., 1986). Thus, generally, MTX exhibits an initial rapid distribution phase characterized by binding to elements of the blood (erythrocytes, leukocytes, and platelets), an intermediate distribution phase, and a relatively slow elimination.

MTX has a described distribution value of up to 2248 L/m^2 (Scott and Figgitt, 2004). The large volume of distribution of MTX is due to the sequestration and accumulation in tissues, as an unchanged drug (Ehninger et al., 1985, Batra et al., 1986, Ehninger et al., 1990, Fox, 2004). MTX is mostly bound to human plasma proteins (approximated 78%) and the extent of binding is not affected by the presence of others drugs, like DOX (Batra et al., 1986).

MTX is typically found at high concentrations in highly perfused tissues such as liver, thyroid, spleen, and heart, and can remain in the body up to 272 days (Ehninger et al., 1990). The elimination of MTX of the organism occurs by hepatobiliary and renal route as unchanged drug or as metabolites (monocarboxylic and dicarboxylic acid derivatives) originated from oxidation of the terminal hydroxyl groups of the side chains, as well as glucuronide conjugates of these acids. Other metabolites were also identified, such as acetoxy ester derivative, glutathione conjugates, and naphthoquinoxaline (Rossato et al., 2013b). The MTX metabolism is not fully understood, although it is known that its clearance is reduced in patients with hepatic dysfunction (Ehninger et al., 1990, Fox, 2004, Seiter, 2005).

Table 3 - Details of therapeutics and common toxicities of DOX and MTX

Drug	Usual dose	Treatment	Common toxicities
Doxorubicin	60 - 75 mg/m^2	Solid tumors Lymphoma Myeloma	Myelosuppression Mucositis Cardiac toxicity Extravasation risk
Mitoxantrone	5 - 12 mg/m^2	Prostate cancer Acute leukemia Multiple sclerosis Other solid tumors Lymphoma	Myelosuppression Mucositis Cardiac Toxicity Extravasation Risk

Adapted from (Seiter, 2005).

1.5.2.2. Cardiotoxicity of mitoxantrone

MTX-induced cardiotoxicity is clinically similar to anthracyclines and includes arrhythmias, decreased LVEF, CHF, tachycardia, electrocardiogram (ECG) changes, and myocardial infarction (Pai and Nahata, 2000). These cardiac events may occur in up to 18% of patients receiving MTX (Seiter, 2005). The incidence of cardiac effects of MTX increases with the total cumulative dose. MTX is administered at a lower relative dose when compared to anthracyclines, in a conversion dose approximately of 4.5 mg of DOX to 1 mg of MTX (Seiter, 2005). Presently, the recommended maximum lifetime cumulative dose of MTX is 140 mg/m^2, with 2.6 to 13% of patients developing cardiac toxicity in that dose. However, in multiple sclerosis patients, there are reports of cardiac toxicity at a cumulative dose of 100 mg/m^2 (Seiter, 2005, Namaka et al., 2011). A cumulative dose of 85 mg/m^2 was already considered above the safety limit for patients with no cardiac risk factors, however, even lower cumulative doses have shown toxic effects on myocardial cells (Villani et al., 1989). Thus, it is crucial that even patients receiving a cumulative dose lower than 85 mg/m^2 should be carefully monitored (Shpall et al., 1988, Villani et al., 1989).

As early as the 80's, the cardiotoxicity of MTX has been reported. Thirty-five patients with hepatocellular carcinoma were treated with MTX. Cardiac events occurred in 5 patients, 3 of whom had received high total cumulative dose of MTX (ranging between 126 - 134 mg/m^2). One of the patients had previous therapy with DOX (cumulative dose of 480 mg/m^2), while the fifth patient suffered left bundle branch block with only 12 mg/m^2 cumulative dose (Dunk et al., 1985). In 1987, forty-six patients with acute leukemia were treated with MTX as a single agent. A dose of 12 mg/m^2 of MTX was given i.v. on five consecutive days. Cardiotoxicity was documented in 7 patients: 6 of these patients had previous therapy with anthracyclines, while the seventh patient had a total MTX dose of 235 mg/m^2 with no concomitant or previous anticancer therapy (Vorobiof et al., 1987).

In a retrospective study including all patients (163 patients) with multiple sclerosis treated with MTX, 14% developed *de novo* cardiotoxicity (evaluated by decreased LVEF) by the end of the study (Kingwell et al., 2010). Also in a multiple sclerosis study, 20 of the 31 patients prematurely ended the treatment due to cardiac abnormalities or quality of life issues. The reasons for therapy cancelation related to cardiac complications accounted for 32%, ranging from moderate (heart palpitations, tachycardia) to severe (LVEF decreased by >10% from baseline and / or >50%) cardiac abnormalities (Namaka et al., 2011). These same authors have shown that patients who discontinued MTX-treatment due to cardiac events had post-treatment reversion to normal cardiac function after discontinuation, which was also demonstrated by Paul *et al.* (2007).

All these case reports clearly demonstrate that MTX is a highly cardiotoxic therapeutic agent and that higher cumulative doses and previous or concomitant cardiotoxic therapy largely increases the cardiotoxicity.

1.5.2.3. Mitoxantrone and its mechanisms of cardiotoxicity

Some cardiotoxic effects of MTX include arrhythmias, decreased LVEF, CHF, tachycardia, ECG changes, and myocardial infarction (Pai and Nahata, 2000). The mechanisms of MTX-induced cardiotoxicity are poorly understood. MTX was thought to share the same biochemical mechanisms of anthracycline-induced cardiotoxicity, since DOX and MTX share clinical aspects of cardiac toxicity. However, their cardiotoxicity mechanisms seem to be different in several aspects. MTX is not very prone to induce ROS formation (Novak and Kharasch, 1985). In fact, MTX was able to inhibit endogenous or drug-stimulated lipid peroxidation in cardiac sarcosomes and mitochondria, interrupting lipid hydroperoxide-dependent initiation and propagation reactions in the peroxidative cascade (Novak and Kharasch, 1985, Ehninger et al., 1990). In primary cultures of cardiomyocytes from adult rats, MTX reduces cell viability and the number of rod-shaped cells to the greatest extent, followed by carminomycin, idarubicin, and epirubicin (Andersson et al., 1999). MTX has the highest lipophilicity of the molecules tested. In fact, MTX is highly lipophilic and is found in high concentrations in the heart after administration (Ehninger et al., 1985).

Using electron microscopy, MTX-treated cardiomyocytes isolated from 1- to 2-day-old neonatal rats showed disorganized myofibrillar structures, swollen mitochondria and sarcoplasmic reticulum, extensive vacuolization, as well as discontinuous plasma membrane (Shipp et al., 1993). MTX also has effects on the energetics of cardiomyocytes, particularly causing reduction of cellular ATP levels in a dose- and time-dependent manner, and also decrease in the spontaneous synchronous beating in isolated cardiomyocytes (Shipp et al., 1993). MTX, as DOX, inhibits cellular respiration (Cini-Neri and Neri, 1986) and impairs cardiac respiratory control, causing cellular ATP depletion (Shipp et al., 1993). Mitochondrial dysfunction can be responsible for oxidative stress and MTX seems to interfere with its bioenergetics and, thus, trigger mild oxidative stress (Costa et al., 2013b). This fact was demonstrated in H2c9 myocyte cell lines incubated with MTX, in which the ROS increase was only detected after ATP and ATP synthase compromise (Rossato et al., 2013c).

Studies in animals, isolated neonatal rat heart myocytes and lately in humans showed that the use of the iron-chelator, dexrazoxane, may be cardioprotective, highlighting the pro-oxidant ability of MTX in the heart via iron pathways (Herman et al., 2001, Bernitsas et al., 2006). In HL-1 cardiac cell line, MTX was able to increase oxidized glutathione (GSSG) and

decrease the total glutathione levels, evidencing that MTX has the ability to disturb antioxidant defenses (Costa et al., 2013a). Thus MTX induces high cardiac damage, in spite of its low capacity to induce oxidative stress and lipid peroxidation (Novak and Kharasch, 1985, Arnaiz and Llesuy, 1993).

MTX is resistant to reductive enzymatic activation but, in the presence of high concentration of H_2O_2, which happens in heart even in non-stressful circumstances, or by oxidative enzymatic activation through microsomal or peroxidase enzymes, the metabolism of MTX may involve oxidation processes, following by conjugation with reduced glutathione (GSH) or glucuronic acid (Mewes et al., 1993, Costa et al., 2013b). The nucleophilic addition of the thiol group of GSH leads to the formation of the thioether conjugates of MTX, demonstrating the propensity of the oxidatively activated MTX to bind covalently to the thiol of peptides (Mewes et al., 1993). Moreover, the metabolism of MTX can be related to its cardiotoxicity. The cytotoxicity caused by extracts of S9 liver fractions that metabolized MTX was higher in the H9c2 cells than what was observed with liver fractions that did not metabolized MTX (Rossato et al., 2013b). Furthermore, in the same study, the co-incubation of MTX with CYP450 and CYP2E1 inhibitors partially prevented the cytotoxicity observed in the MTX group incubated in the same myoblast model (H9c2), highlighting that MTX metabolism is also relevant for its undesirable effects (Rossato et al., 2013b).

Collecting all the scarce data available, it is known that, *in vivo*, MTX is not able to cause an initial redox cycle to trigger an oxidative stress state. Instead, MTX and naphthoquinoxaline, an active metabolite of MTX that has been identified in animal and human models (Blanz et al., 1991, Rossato et al., 2013b), easily accumulate in the heart, impairing iron pathways and ATP homeostasis (Shipp et al., 1993). Although MTX seems to have the same clinical cardiotoxicity profile of anthracyclines, their mechanisms of cardiotoxicity differ and MTX cardiotoxic mechanisms remain largely unknown, and little is known about the risk of the pediatric population to develop heart toxicity after MTX treatment.

1.6. Pediatric oncology and cardiotoxicity

Throughout the years, research in the chemotherapy field has yield several breakthroughs regarding pediatric cancer treatment: before the 1970s, the 5-year survival in children was lower than 50% (Scully and Lipshultz, 2010), while nowadays it reaches 70% in the USA and Western Europe (Lipshultz et al., 2008). Only in the USA, the population of long-term survivors of pediatric cancers is estimated to be more than 360 000 (Scully and Lipshultz, 2010). Anthracyclines are very commonly used in the treatment of childhood

leukemia and solid tumors (Kremer et al., 2002) and it is estimated that more than 50% of all childhood cancer survivors have been treated with anthracycline-based therapy (Chen et al., 2011, Dillenburg et al., 2013).

Although cancer recurrence and secondary malignancies are concerning problems in children cancer survivors, children have a long life-expectancy after a successful antineoplastic treatment and thus it is important to have a follow-up protocol for pediatric patients. Cardiovascular diseases are important risk factors influencing long-term survival, having children higher mortality risk when compared to the general population (Lawless et al., 2007, Dillenburg et al., 2013). As stated before, in a study from the Childhood Cancer Survivor Study in 10397 children diagnosed with cancer in the 70s and 80s and treated with anthracyclines or non-anthracyclines based regimen, it was observed that childhood survivors were at 15.1-fold higher rate of developmental of CHF and 10.4-fold higher rate of developmental of cardiovascular disease when compared with siblings (Oeffinger et al., 2006).

1.6.1. Cardiotoxicity in pediatrics with anthracyclines

Late cardiotoxicity is more frequent than early cardiotoxicity in children treated with anthracyclines (Krischer et al., 1997, Seiter, 2005). In general, the younger the patient, the greater the susceptibility to develop cardiac damage. The age when patients receive treatment with anthracyclines seems to be a crucial factor for the development of cardiotoxic events, since inadequate ventricular growth occurs in time (Krischer et al., 1997). The risk of developing anthracycline-induced CHF is 9.8% if children are treated with a cumulative anthracycline dose of 300 mg/m^2 or more, 20 years after the start of anthracycline therapy (van Dalen et al., 2006). Children receiving cumulative doses of anthracyclines >550 mg/m^2 have a cardiotoxicity risk 5 times higher of developing cardiotoxicity compared to the risk among those receiving lower cumulative doses (Krischer et al., 1997). Abnormalities of cardiac structure and function have been registered in 65% of survivors of childhood acute lymphoblastic leukemia, 6 years after the end of therapy with anthracyclines (Lipshultz et al., 1991).

Although adults usually develop chronic dilated cardiomyopathy (significantly reduced left ventricular fractional shortening and contractility with left ventricular dilation) after anthracycline therapy, children typically develop dilated cardiomyopathy and/or restrictive cardiomyopathy (Lipshultz et al., 2008), characterized by diastolic dysfunction and normal to reduced left ventricular dimension with significantly reduced left ventricular thickness, fractional shortening, and contractility (Lipshultz et al., 2005). Late symptomatic

cardiomyopathy in 15 of 300 patients that ended anthracycline-based treatment with a median of 11 years-old were also reported (Steinherz et al., 1995). These same patients had a median of 22 years-old at the time of late cardiac decompensating and one with symptomatic anthracycline cardiotoxicity had heart transplantation.

When looking at subclinical / asymptomatic cardiotoxicity, namely abnormal systolic function and/or increased afterload, the reported frequency ranged between 0-57% in a review of studies between 1966 and 2001 including children (newborn infant to 18 years-old) treated with anthracyclines (Kremer et al., 2002). The exact incidence percentage is difficult to determine because of the lack of standardized detection and reporting of cardiovascular events, as well as of insufficient long-term follow-up (Chen et al., 2011).

Anthracyclines in growing children seem to alter heart growth potential and its compensatory mechanisms (Urbanova et al., 2010). These same mechanisms were already demonstrated in laboratory with juvenile mice, which showed depletion of the cardiac progenitor cells that are essential to regeneration and blood vessel formation, when exposed to anthracyclines (Huang et al., 2010). A cumulative dose of 3 mg/kg of DOX in postnatal mice reduced the size of the cardiac progenitor pool, impaired their ability to differentiate into cardiac and vascular cells lineage and affected vascular development in the heart (Huang et al., 2010). It was possible to show, in rats, that DOX impaired progenitor cells and caused heart failure. The exogenous delivery of progenitor cells counteracted the progression of DOX-cardiotoxicity: cardiac progenitor cells were injected in the failing myocardium and this treatment promoted regeneration of cardiomyocytes and vascular structures, improving ventricular performance, and animal survival (De Angelis et al., 2010). In a recent study, CHF was the most common cardiac disturbance (54% of the total cardiac events), followed by cardiac arrhythmia, ischemia/myocardial infarction, valvular disease and pericarditis in childhood cancer survivors that were treated with anthracyclines and/or cardiac irradiation (van der Pal et al., 2012).

1.6.2. Cardiotoxicity in pediatrics with mitoxantrone

MTX is used in pediatric population, either in cancer treatment as a second line therapy of multiple sclerosis (van Dalen et al., 2004, Kornek et al., 2011). MTX-pediatric users and survivors will largely increase in the coming decades with increased incidence of MTX-induced cardiotoxicity (Ungerleider et al., 1985, van Dalen et al., 2004). The younger age (< 4 years old), concomitant cardiotoxic therapy, and other cardiac comorbidities largely increase MTX-cardiotoxicity incidence, but, so far no mechanisms have been discovered (Chen et al., 2011).

In a review of 17 studies done in 2004 by van Dalen and co-authors, they estimated that MTX-related symptomatic cardiotoxicity / clinical heart failure varies between 0 and 6.7% and asymptomatic cardiac damage varies between 0 and 80% in children (van Dalen et al., 2004). However, the exact cumulative incidence is difficult to estimate due to the low methodological quality, namely definitions of subclinical cardiotoxicity and differences in the clinical data, namely regarding to the patients, to the dose of the drugs, which does not permit to know if MTX is less cardiotoxic than anthracyclines in children (van Dalen et al., 2004, Ojha et al., 2013).

Developmental of irreversible cardiac dysfunction in one child with acute leukemia requiring heart transplant was described several years after therapy with an anthracycline and anthracenedione (Urbanova et al., 2010). The 4 year-old boy was treated with DNR and MTX and 12 years after the diagnosis, he developed symptoms of cardiac dysfunction, including decreased LVEF, mitral insufficiency, dilated cardiomyopathy, and CHF. He required a heart transplant 19 years after the diagnosis (Urbanova et al., 2010).

In the treatment of advanced acute leukemia and solid tumors in 84 children, 6 developed evidence of cardiac dysfunction, including 3 occurrences of CHF (Ungerleider et al., 1985). In a study with 101 patients with advanced pediatric malignant solid tumors, 2 died due to CHF after receiving 54 and 90 mg/m^2 of MTX, and previous DOX treatment, and 3 had changes in cardiac function (Pratt et al., 1986). In another study of the same author, 2 children receiving MTX that were previously treated with DOX and cisplatin, were reported to develop CHF. One of the patients died two weeks later (Pratt et al., 1983). A report by O'Brien *et al.* that included children with Down syndrome and newly diagnosed with acute myeloid leukemia (n = 57) treated concomitantly with DNR and MTX, indicate 10 cases of development of symptomatic cardiomyopathy during or soon after completion of treatment and, of these, 3 deaths resultant of CHF (O'Brien et al., 2008). Also following the concomitant treatment (cytosine-arabinoside and MTX) of 19 children with acute myeloid leukemia, one died after further MTX treatment due to toxic cardiomyopathy (Ritter et al., 1987). In 2007, in a study with 34 children with acute myeloid leukemia ranging from 2 months to 15 years-old, two that received cumulative doses of at least 400 mg/m^2 of anthracycline equivalents (DNR and MTX) experienced symptomatic heart failure; and 5 from other dose protocols experienced asymptomatic left ventricular systolic dysfunction (Tan et al., 2007). Also in children treated for acute myeloid leukemia, Dahl *et al.* reported cardiotoxicity manifested by decreased LVEF or shortening fraction in 15 of a total 66 patients, with 3 episodes of CHF occurrence, when treated with combination of MTX, etoposide, and cyclosporine (Dahl et al., 2000).

At this point, no mechanisms were attributed to the MTX cardiotoxicity in young patients.

2. AIMS OF THE STUDY

Is the anti-tumor drug mitoxantrone more cardiotoxic in pediatric than in adult mice?

The cardiotoxicity induced by MTX has long been described. Furthermore, the quality of life of cancer survivors is a matter of concern (van Dalen et al., 2004, Seiter, 2005). Despite the widespread use of MTX and the fact that it presents a toxic cardiac clinical profile similar to DOX, their mechanisms of toxicity towards non-target tissues, namely heart, are different and the mechanisms of MTX-induced cardiotoxicity remain largely unknown (Menna et al., 2008b). Cardiac damage promoted by MTX is a serious life-threatening issue as it increases mortality and morbidity, and the heart is very susceptible to this chemotherapeutics agent (Seiter, 2005). Additionally, MTX is used for the treatment of several tumors, including childhood cancers such as lymphoma and leukemia, as well as multiple sclerosis, which makes MTX-cardiotoxicity a concerning issue also in this population. Children that survive cancer can potentially have a long life-expectancy after treatment, but little is known about the risk of this population to develop heart toxicity after MTX treatment. Only a better understanding of the mechanisms involved in the injury caused by chemotherapeutics to normal tissues will allow the designing of better therapies. In this context, the present study was undertaken to investigate what is the impact of MTX-induced cardiotoxicity in childhood and which parameters are associated to this toxicity. So far, no laboratory studies were done with MTX in pediatric populations. This dissertation aims to seek plausible explanations for MTX-induced cardiotoxicity and susceptibilities in young animals. Since the cumulative dose is the most predictive risk factor for cardiotoxicity, different MTX cumulative dose were used. Moreover, multiple administration of MTX, interrupted by free-drug periods, was given to mimic the human MTX-therapy in pediatric and adult CD-1 mice, which were sacrificed weeks after the last administration in order to evaluate cumulative toxicity. Additionally, liver and kidneys were also studied. To achieve the aims, several techniques were used, namely enzymatic assays to evaluate the redox status (GSH/GSSG), bioenergetics (ATP) and apoptotic events in tissues. Plasma clinical parameters (AST, ALT, total-CK and CK-MB) in plasma and lipid peroxidation in tissues, as well as histopathological examination of the heart were also performed.

3. MATERIALS AND METHODS

3.1. Chemicals

Sodium phosphate monobasic was purchased from Panreac (Barcelona, Spain), potassium sodium tartrate to Fluka (Buchs SG, Switzerland), and HEPES and sodium chloride to VWR (Leuven, Belgium). Ammonium acetate, dimethyl sulfoxide (DMSO), EDTA, Folin–Ciocalteu reagent, Histosec paraffin pastilles, magnesium chloride, perchloric acid, trichloroacetic acid (TCA), sodium hydroxide, copper (II) sulfate, sodium carbonate, disodium phosphate, were purchased from Merck (Darmstadt, Germany). Methanol, acetonitrile, and xylene were purchased from Fisher Scientific (Loughborough, UK). The peptide substrate for caspase-3 (Ac-DMQD-AMC), the peptide substrate for caspase-8 (Ac-IETD-AMC), and the peptide substrate for caspase-9 (Ac-LEHD-AMC) were obtained from Peptanova (Sandhausen, Germany). Eosin 1% aqueous was obtained from Biostain (Traralgon, Australia), Harris hematoxylin was from Harris Surgipath (Richmond, IL, USA) and Histofluid from Marienfeld (Lauda-Königshofen, Germany). All the other reagents used in this dissertation were purchased from Sigma-Aldrich at the highest purity available (St. Louis, MO, USA).

3.2. Animals

Male CD-1 mice weighing 10-12g and 38-40g were obtained from Charles River Laboratories (L'Arbresle, France) in experiment 1 and from Harlan (Udine, Italy) in experiment 2 and kept in the vivarium of Instituto Superior de Ciências da Saúde – Norte (ISCS-N) – CESPU (Paredes, Portugal). They were let to adjust to the environmental conditions for 4 days, before experiments began. The animal weighing 10-12g, corresponding to a pediatric population had approximately 3-weeks old and the mice with 38-40g were approximately 8 to 10 weeks-old. According to the literature available, the first group did not enter puberty (child) while the latter is in adulthood, being considered a suitable approach to perform the experiments and mimic human MTX-therapy in pediatric and adult populations (Eisen, 1976, Østergaard et al., 2011).

All animal procedures were performed focusing on improving animal welfare. The laboratory animal research was performed with awareness to the various issues regarding the three Rs and the present legislation, which meet the research ethics concepts of Replacement, Refinement, and Reduction (Costa and Antunes, 2011, Olsson et al., 2011). The animals were housed in a cage 1290D Eurostandard Type III (425 x 266 x 155 mm - floor area 820 cm^2) in a temperature (22 ± 2ºC) and humidity-controlled environment and a

12h light-dark cycle. Standard rodent chow 4RF21 GLP certificate diet (Mucedola, Settimo Milanese, Italy) and water were provided *ad libitum*. Housing and experimental treatment of the animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (ILAR 1996) and with the guidelines defined by the European Council Directive (86/609/EEC). The number of animals per cage was according to European Union recommendations and revision of Appendix A of the European Convention ETS 123 (Figure 9). Considering the time of the animal experiments and the probable growth of the animals, a maximum of 8 animals *per* cage was determined. This allowed socialization and acceptable welfare conditions. Moreover, animal experiments were licensed by the Portuguese General Directory of Veterinary Medicine (reference number 0421/000/000/2013) and approved by the Ethical Committee of Faculdade de Farmácia da Universidade do Porto (protocol number 7/03/2013).

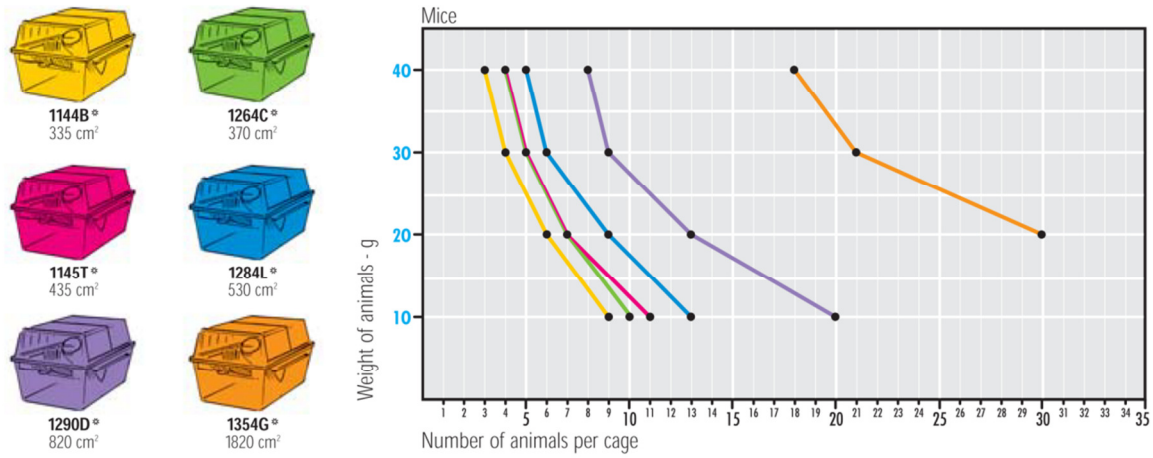

Figure 9 - Number of animals per cage according to EU recommendations and Revision of Appendix A (ETS 123) (Tecniplast, 2009).

3.3. Study design

The administration schedule of MTX was given in order to mimic the human MTX-therapy that consists of multiple administration in separated time-points (Vorobiof et al., 1987, Paul et al., 2007). All injections were given at afternoon to improve tolerability of high-dose of MTX, as seen in the literature (Levi et al., 1994). Consumption of food and water, and animal weight were recorded along the experiments, twice a week. Animals were kept in a social environment as a group (Curfs et al., 2011) and, therefore, food and water consumption were assessed for the entire group and afterwards calculated in function of body weight of each animal.

3.3.1. Experiment 1

The animals were divided into three groups of pediatric mice (A, B, C) and three groups of adult mice (D, E, F), of 6 animals each (Figure 10). Animals in groups B and E were subjected to a total cumulative dose of 4.5 mg/kg MTX as a result of 6 intraperitoneal (i.p.) injections (2 *per* week). To animals in groups C and F was given a total cumulative dose of 9.0 mg/kg MTX as a result of 6 i.p. injections (2 *per* week). Animals in groups A and D were the respective controls and were given NaCl 0.9 % (saline solution) in the same schedule (Figure 10). In the cumulative dose of 9.0 mg/kg MTX, treated mice received each time 1.5 µg/g body weight of MTX dose solution (0.1 mg/mL MTX) and in the cumulative dose of 4.5 mg/kg MTX, treated mice received, each time, 0.75 µg/g body weight of MTX solution (0.05 mg/mL). To control animals was given 0.9% saline solution in the same equivalent volume of both treated-conditions. The injections were given on the left side of the peritoneum in the 1st, 2nd and 5th injection days, and on the right side in the 3rd, 4th and 6th, using disposable 1 mL sterile U-100 insulin syringes and a 25 gauge (G) Neolus needle (Terumo Europe España, Madrid, Spain). MTX dihydrochloride was dissolved in sterile 0.9% saline solution. After the last MTX administration, the animals were maintained in a drug-free period for 20 days to allow the development of cumulative toxicity, before sacrifice.

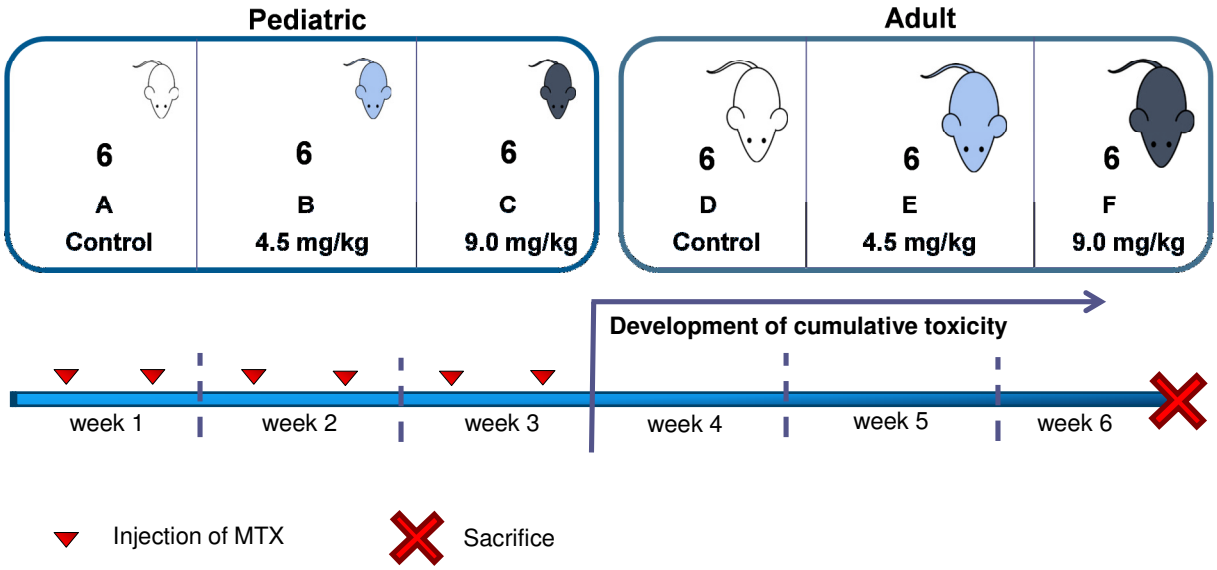

Figure 10 - Schematic representation of the distribution of the animals by age group and doses (control, cumulative doses of 4.5 mg/kg or 9.0 mg/kg of MTX), as well as the timeline of the administration of MTX and time to sacrifice.

3.3.2. Experiment 2

The animals were divided into four groups of pediatric mice (A, B, C, D) and four groups of adult mice (E, F, G, H), of 8 animals each. All animals were given 6 i.p. injections (2 *per* week) following the same schedule and procedure as described in experiment 1. Animals in

groups B and F were given a total cumulative dose of 7.0 mg/kg MTX. Animals in groups D and H were given a cumulative dose of 9.0 mg/kg MTX. Animals in groups A/E and C/G were the controls of the dose of 7.0 mg/kg MTX and 9.0 mg/kg MTX, respectively, and were given NaCl 0.9 % (Figure 11). The cumulative dose of 9.0 mg/kg MTX-treated mice received 1.5 µg/g body weight of MTX dose solution each day (0.1 mg/mL MTX) and the cumulative dose of 7.0 mg/kg MTX-treated mice received 1.17 µg/g body weight of MTX solution (0.08 mg/mL). To control animals were given 0.9% saline solution in the same equivalent volume. After MTX administration, the animals dosed 9.0 mg/kg of MTX were sacrificed 24h after the 6th and last injection, whereas the animals of cumulative dose 7.0 mg/kg of MTX were maintained in a drug-free period, in order to allow the development of cumulative toxicity, before sacrifice. According to animal welfare and data gathered in experiment 1, these animals were sacrificed at day 14 after the last administration to avoid further suffering and loss of animal samples.

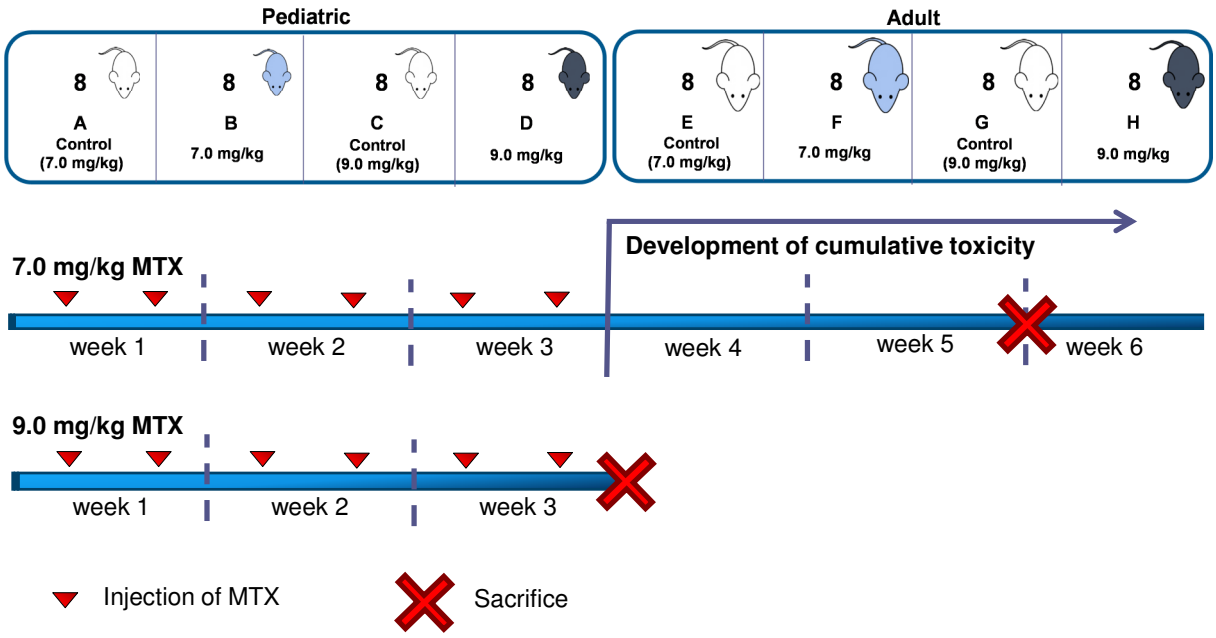

Figure 11 - Schematic representation of the distribution of the animals by age group and cumulative concentrations (cumulative doses of 7.0 mg/kg or 9.0 mg/kg of MTX and respective controls), as well as the timeline of the administration of MTX. Oppositely to the cumulative dose of 7.0 mg/kg of MTX, no time was given to the dose of 9.0 mg/kg of MTX-treated animals for development of cumulative toxicity and sacrifice was done 24h after the last administration.

3.4. Blood and tissue collection

The animals were anesthetized with isoflurane inhalation and sacrificed by exsanguination, collecting the blood in inferior vena cava into EDTA-containing tubes. This blood was used for the determination of plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine-kinase MB (CK-MB) and total creatine kinase (total-CK).

Hematological parameters (neutrophils, eosinophils, basophils, lymphocytes, monocytes, red blood cells, hemoglobin, and platelets) of two mice of each group were also determined. These determinations were performed in an external laboratory as described in the following section.

Immediately after sacrifice, the heart was excised, separated from the pericardium, and weighed. In experiment 2, a transverse section (approximately 2mm from the apex) on the heart of three mice of each group was collected and fixed in 4% paraformaldehyde (diluted in PBS 1X, 2.5% sucrose, 0.1% glutaraldehyde, pH 7.2-7.4), for examination of cardiac damage by light microscopy. The same section from the remaining animals was collected and frozen at -80ºC for future western blot analysis. Furthermore, approximately 40mg of the heart was collected in complete lysis buffer (25 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 1 mM pefabloc, 5 mM DTT, pH 7.4) to measure the activities of caspases 3, 8, and 9 and frozen a -80 ºC until the assays. The remaining heart was homogenized with a Potter homogenizer in a phosphate buffer KH_2PO_4 100mM (pH=7.4). The animals' liver and kidneys were also removed, weighed, and homogenized with an Ultra-Turrax homogenizer in the same phosphate buffer. A homogenate aliquot of the organs was treated with 10% perchloric acid 1:1 (5% final acid concentration) to precipitate proteins and centrifuged at 13000 rpm for 10 min at 4ºC. The resulting supernatant was separated and frozen at -80ºC for ATP determination and at -20ºC for GSHt/GSSG determinations. Another aliquot of the homogenates was mixed with 10% trichloroacetic acid 1:1 (5% final acid concentration), supplemented with 10 µL of 5% of antioxidant butylated hydroxytoluene (BHT) (diluted in methanol) for each mL of the mix and immediately frozen at -80ºC (for a maximum one month) until determination of malondialdehyde (MDA) levels. The remaining homogenates were frozen at -20ºC for protein determination. All these procedures were performed in ice-cold tubes.

3.5. Measurement of hematological parameters, aminotransferases, total-CK and CK-MB

After blood collection in EDTA-containing tubes, the hematological parameters were quantified in the day of the experiments using an automatic blood cell counter ABX Micros 60 (Horiba ABX, Amadora, Portugal). The remaining blood was centrifuged at 920*g* for 10 min for separation of plasma. The plasma was frozen at -20ºC until determination of AST, ALT, CK-MB and total-CK. These determinations in the mice plasma were done through enzymatic assays in the apparatus ABX Pentra 400 with ABX Pentra reagents (Horiba ABX, Amadora, Portugal), according to the manufacturer's instructions. The hematological

determinations were performed by Drª Bárbara Duarte and the biochemical parameters by Drª Laura Pereira, in Unidade de Análises Clínicas at Faculdade de Farmácia da Universidade do Porto

3.6. Determination of total glutathione (GSHt) and GSSG

The GSHt and GSSG contents of tissue homogenates were determined by the 5,5′-dithiobis(2-nitrobenzoic acid) DTNB-GSSG reductase recycling assay (Costa et al., 2007, Costa et al., 2009). For the GSHt assay, 200 µL of acidic sample supernatant / standards were neutralized with 200 µL of 0.76 M $KHCO_3$, vortexed, and centrifuged for 2 min at 13000 rpm (4ºC). The reagent solution, containing 0.68 mM NADPH and 3.96 mM DTNB, was prepared in phosphate buffer (71.5 mM Na_2HPO_4, 71.5 mM NaH_2PO_4 and 0.63 mM EDTA) before the measurements and kept protected from light. For quantification of GSHt, 100 µL of neutralized samples, standards and blank were added in triplicate to 96-well microtiter plate, followed by addition of 65 µL/well of the reagent solution and incubation in a Biotek PowerWaveX plate reader (Winooski, VT, USA) for 10 min at 30ºC. Then, 40 µL of glutathione reductase 10 IU/mL in phosphate buffer was added per well. The formation of 5-thio-2-nitrobenzoic acid (TNB) was monitored by 10 seconds intervals for 3 min at 415 nm. For the quantification of GSSG, 10 µL of 2-vinylpyridine was added to 200 µL of the acidic supernatant and mixed for 1 h in ice to block GSH. Then, the determination of GSSG was performed as described above for GSHt. The GSH was calculated using the following formula GSH = GSHt – 2 x GSSG.

3.7. Assessment of lipid peroxidation

The assessment of lipid peroxidation in the tissue samples was performed by measuring the MDA values and comparing them with a standard curve of MDA after reaction with thiobarbituric acid (TBA). The analysis of MDA-equivalent content in standards and samples was performed by high-performance liquid chromatography with diode-array detection (HPLC-DAD) in experiment 2 (Ying et al., 2008) and by a fluorescence-based microtiter plate assay in both experiments (Dhiman et al., 2013) with few modifications. The standard solution of MDA was freshly prepared in a final concentration of 15mM in 5% TCA. The calibration curve varied from 0.15 µM to 6 µM MDA in 5% TCA. The TBA derivatization was performed in to order to allow the formation of a complex between MDA and TBA. Two hundred µL of 0.8% TBA were added to 200 µL of blank / standards / samples and incubated at 80ºC for 60 min for derivatization and then cooled down.

The tissue samples were centrifuged at 13 000 rpm for 5 min (4ºC), to allow protein precipitation and remove as many interfering compounds as possible, since TBA reacts with a variety of non-MDA compounds.

3.7.1. HPLC-DAD determination

After the reaction between TBA and MDA-free content in samples and standards was performed, the quantification of the complex was performed by HPLC on a Waters 2690 separation module (Waters, Milford, MA, USA), with a photodiode array detector Waters 996 set to record between 500 and 600 nm wavelengths. A commercially pre-packed reverse phase cartridge of 250 mm × 4.6 mm, Waters Spherisorb RP-18 (5 µm) ODS2 column was used. The chromatographic and spectral data were processed by Millenium[32] software (Waters). Samples were injected by isocratic elution (1 mL/min) with ammonium acetate aqueous solution (10mM, pH=6.8):acetonitrile (80:20). The injection volume was 100µL for heart samples and 20 µL for liver and kidneys samples. The analysis was completed within 6 min.

3.7.2. Fluorescence microplate determination

The remaining volume of the derivatized standards and samples was used for fluorescence assay in a microplate reader. One hundred µL from each reaction was loaded to 96-well black microtiter plate in duplicate and the fluorescence ($\lambda_{excitation/emission}$ = 530nm/590nm) was read on microplate reader Biotek Synergy HT (Winooski, VT, USA).

3.8. Determination of cellular ATP levels

The ATP levels of heart, liver and kidneys were determined by bioluminescence through the reaction with the firefly luciferin-luciferase system (Costa et al., 2007). D-Luciferin 90.9 mg/L stock reagent and luciferase from *Photinus pyralis* (firefly) (3 000 000 U/mL final concentration) were prepared in luciferin-luciferase buffer (50mM glycine, 10mM $MgSO_4$, 1mM Tris, 0.55 mM EDTA, 0.1% bovine serum albumin, pH = 7.6) and light protected aliquots were stored at -20ºC until use. Before the assay, luciferin-luciferase reaction system was constituted by adding the two solutions and then kept at room temperature. Briefly, the assay consisted of neutralizing 150 µL of samples, standards or blank with 150 µL of 0.76 M of $KHCO_3$, followed by vortex, and centrifugation for 10 min at 13000 rpm (4ºC). For quantification of cellular ATP levels, 100 µL of neutralized supernatants were added to 96-well white microtiter plate, followed by addition of 100 µL/well of the luciferin-luciferase reagent and read immediately in the Biotek Synergy HT (Winooski, VT, USA) plate reader.

Light output was given as the integral relative light units. The assay was made sequentially with few measurements in each reading in order to avoid loss of bioluminescence signal.

3.9. Determination of caspase-3, -8 and -9 activities

A portion of the heart (~40mg) was homogenized with a Potter A561 in 400 µL lysis buffer and then the obtained lysate was centrifuged at 13000 rpm for 30 min at 4ºC (Darwish et al., 2012). The measurement of caspase-specific enzymatic activity was assessed by fluorescence with some modifications (Maianski et al., 2004). The hydrolysis of the peptide substrate Ac-DMQD-AMC by caspase-3, of the peptide substrate Ac-IETD-AMC by caspase-8, and of the peptide substrate Ac-LEHD-AMC by caspase-9 results in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety, which has a excitation and emission maximum wavelength at 360 nm and 460 nm, respectively. For each caspase activity assay, 200 µL of assay buffer (100 mM NaCl, 50 mM HEPES, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, 10mM DTT pH 7.4) was placed in a black 96-well microtiter plate, followed by 40 µL of the tissue lysate and 10 µL of the respective caspase substrate. For negative control, substrates were mixed with 250 µL of complete assay buffer. The final concentration of each substrate in the assay buffer was 100 µM. Microtiter plates (light protected) were softly shaken and incubated at 37ºC for 24h. The fluorescence was read in the Biotek Synergy HT ($\lambda_{excitation/emission}$ = 360nm / 460nm). The enzymatic activities were normalized to the protein content of the tissue lysate and were expressed as units of fluorescence / amount of protein.

3.10. Processing of tissues for optic microscopy

The histological processing of the hearts was performed in the Laboratório de Bioquímica e Morfologia Experimental da Faculdade de Desporto da Universidade do Porto. Histological evaluation was performed in the heart of three mice from each group. The 4% paraformaldehyde-fixed transverse section of the heart was processed as follows: i) fixation in 4% paraformaldehyde [diluted in phosphate buffer solution (PBS) 1X, 2.5% sucrose, 0.1% glutaraldehyde, pH 7.2-7.4] for 10-20 h at 4ºC; ii) rinsed with PBS 1x, pH 7.2-7.4 (overnight at 4ºC); iii) dehydration with 70%, 80%, 95%, 100%, 100% ethanol (60 min each step at room temperature); iv) clearing with xylene (60 min using closed vials at room temperature) and, finally, v) paraffin imbedding with xylene/paraffin 3:1, xylene/paraffin 1:1, xylene/paraffin 1:3, paraffin (twice) (60 min each step using closed vials in an oven at 56ºC). Five µm sections were made with a manual rotary microtome Leica RM2125 (Wetzlar, Germany) that were then stained with hematoxylin and eosin for routine histopathological examination. The

hematoxylin and eosin staining was performed as follows: 5 min xylene (twice), 5 min ethanol 100%, 95%, 80% and 75% (each), 5 min water, 8-10 min hematoxylin, 5 min running water, 4-5 min eosin, 5 min ethanol 95% and 100% (each), 5 min xylene and mounted using Histofluid. These preparations were examined and photographed with a Carl Zeiss Imager A1 light microscope equipped with an AxioCam MRc 5 digital camera (Oberkochen, Germany).

Histopathological evidences of tissue damage were calculated according to their severity and incidence in every slide (Dinis-Oliveira et al., 2007). For each group, at least 15000 cells per slide were analyzed in a blind fashion in order to semi quantify the severity of the following parameters: i) cellular degeneration, ii) interstitial inflammatory cell infiltration, iii) necrotic zones, and iv) loss of tissue organization. The severity of cellular degeneration was scored according to the number of cells showing any alterations (dilatation, vacuolization, pyknotic nuclei, and cellular density) in the light microscopy visual field: grade 0 = no change from normal; grade 1 = a limited number of isolated cells (until 5% of the total cell number); grade 2 = groups of cells (5 to 30% of cell total number); and grade 3 = diffuse cell damage (30% of total cell number). The severity of necrosis was scored as follows: grade 0 = no necrosis; grade 1 = dispersed necrotic foci; grade 2 = confluence necrotic areas; grade 3 = massive necrosis. The inflammatory activity was graded semi-quantitatively into: grade 0 = no cellular infiltration; grade 1 = mild leukocyte infiltration (1 to 3 cells by visual field); grade 2 = moderate infiltration (4 to 6 leukocytes by visual field); and grade 3 = heavy infiltration by neutrophils. The severity of tissue disorganization was scored according to the percentage of the affected tissue: score 0 = normal structure; score 1 = less than one third of tissue; score 2 = greater than one third and less than two thirds; score 3 = grater of two thirds of tissue. For each visual field the highest possible score was 12 and the lowest was 0.

3.11. Protein determination

Protein content was determined by the method described by Lowry, with some modifications (Lowry et al., 1951). Bovine serum albumin was used as protein standard and the calibration curve varied from 25 µg/mL to 250 µg/mL in 0.3 M NaOH. Fifty µL of samples, standards or blank were added in triplicate to a 96-well microtiter plate, followed by addition of 100 µL of extemporaneously prepared Reagent A [9.8 mL of 2% sodium carbonate (Na_2CO_3), 100 µL of 2% sodium potassium tartrate and 100 µL of 1% sulfate cupper II ($CuSO_4$), 98:1:1]. After 10 min light protected, 100 µL of extemporaneously prepared Reagent B (Folin–Ciocalteu reagent and H_2O, 1:14) were added. The microtiter plate was

kept protected from the light for 20 min, after which the absorbance was measured at 750 nm.

The protein content for the caspase activity assay in the tissue lysate was determined using the Bio-Rad RC DC protein assay kit, according to the manufacturer's instructions. Bovine serum albumin was used as protein standard and the calibration curve varied from 200 µg/mL to 1200 µg/mL in 0.3 M NaOH.

3.12. Statistical analysis

Results are presented as mean ± standard deviation (SD). Statistical analysis of weight of animals and their food and water consumptions was carried out using two-way analysis of variance (ANOVA) repeated measurements, followed by Bonferroni *post-hoc* test. When 3 conditions were compared, parametric analysis was performed using the one-way ANOVA when the distribution was normal, followed by Dunn's *post hoc* test or Student-Newman-Keuls *post hoc* test (see details in the legends); or non-parametric analysis was performed with the Kruskal-Wallis test (ANOVA on Ranks) when the distribution was not normal, followed by Student-Newman-Keuls *post hoc* test. A *post-hoc* test was performed when a significant *p* value ($p < 0.05$) was obtained,. When two groups were compared, the t-test was used if distribution was normal, or the Mann-Whitney Rank Sum test if distribution was not normal. Details of the statistical analysis are described in each figure legend. Statistical significance was accepted at $p < 0.05$. Outliers were identified using Grubb's test ($p < 0.05$) only when the data followed a Gaussian distribution. GraphPad Prism 5 software program (San Diego, CA, USA) was used to plot the charts and Sigmaplot 11 software program (San Jose, CA, USA) to perform the statistical analysis.

4. RESULTS

4.1. Experiment 1

4.1.1. Animal survival, body weight and daily food / water consumption

All the adult and pediatric mice receiving the cumulative dose of 4.5 mg/kg of MTX survived with no visible signs of suffering. However, the cumulative dose of 9.0 mg/kg of MTX caused alteration to the overall condition of the animals, since all adult mice (6 animals out of 6) died (Figure 12A) and only 33% of pediatric mice (n = 2) survived until the end of the experiment (day 37) (Figure 12B). These deaths occurred in the first or second weeks after the last i.p. administration of MTX. The animals that died showed sudden signs of suffering (less free movement or response to stimuli) and died within 24 h.

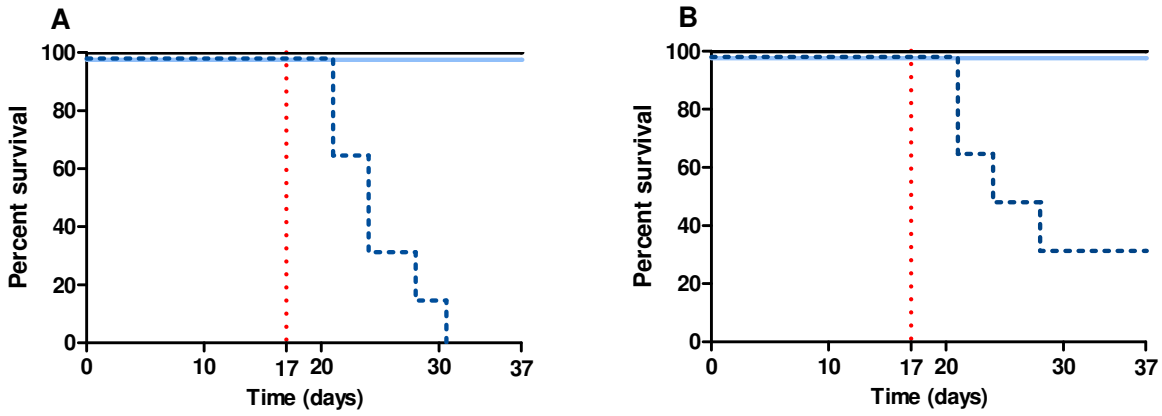

Figure 12 - Survival curves after MTX intraperitoneal (i.p.) cumulative administration in adult (A) and pediatric (B) CD-1 mice. Results are expressed in percent survival. The initial number of the animals is six in each group. Black line (———) represents saline-control treatment, light blue line (———) represents the cumulative dose of 4.5 mg/kg MTX treatment and dashed dark blue line (- - - -) represents cumulative dose of 9.0 mg/kg MTX treatment. The vertical dashed red line represents the last MTX i.p. administration.

In the adult population, the average body weight in animals receiving the cumulative dose of 4.5 mg/kg of MTX was constant since the beginning of the experiment, whereas adults receiving cumulative dose of 9.0 mg/kg of MTX showed a significant weight decrease compared to control (Figure 13A). The difference in body weight between MTX-treated and control mice was statistically significant in adults as soon as the second week after the end of MTX-administration in 4.5 mg/kg-treated animals. In the cumulative dose of 9.0 mg/kg MTX, the loss in weight occurred even sooner (1 week after the last administration). No statistical differences were observed in days 24 and 28 in the highest dose due to the decrease in the number of surviving animals comparing to controls. I was not possible to compare these different *n*. In pediatric population, control and treated groups showed no difference in body weight gain (Figure 13B).

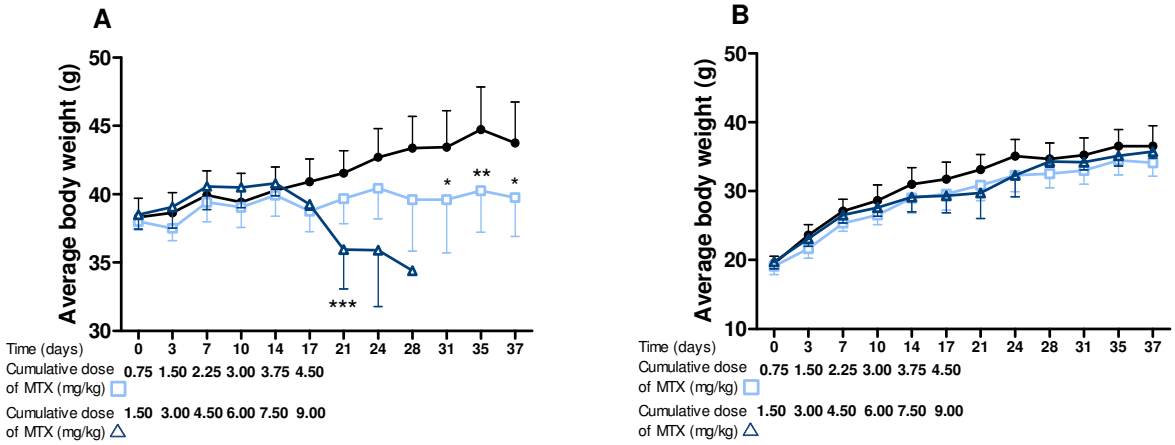

Figure 13 - Average body weight in MTX-treated (exposed to cumulative dose of 4.5 mg/kg MTX and 9.0 mg/kg MTX) and control mice, in adult (A) and pediatric (B). Results in grams (g) are presented as mean ± standard deviation (SD), from six animals in each group, excepting after the 21st day after which the percentage of survival was different from 100%. Light blue open squares (□) represent cumulative dose of 4.5 mg/kg MTX, dark blue triangles (Δ) represent cumulative dose of 9.0 mg/kg MTX and black solid circles (●) represent saline-control. Statistical comparisons were made using two-way ANOVA followed by the Bonferroni *post hoc* test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, treatment vs. control). In 9.0 mg/kg MTX-treated animals, it was not possible to make any statistical comparisons after day 21, since the number of the animals is different from controls.

Regarding food consumption, adults receiving cumulative dose of 4.5 mg/kg MTX had lower consumption since the very beginning of experiment 1, comparing to saline-treated mice. In adult mice receiving cumulative dose of 9.0 mg/kg, food consumption seems to be less constant: in these animals a significant decrease in food consumption after the last administration (17th day) occurred (Figure 14A). In the pediatric mice population, 4.5 mg/kg MTX group had significant lower food consumption comparing to saline-control population. In 9.0 mg/kg MTX group, the difference was more notorious during the MTX administrations; in fact after the last administration the food consumption increased (Figure 14B).

Water consumption data show a decreased intake in adults receiving both cumulative doses of MTX since the beginning of the experiment, but more prominent in highest dose of MTX as time elapsed (Figure 15A). The same occurred in pediatric mice in the 4.5 mg/kg MTX dose. However, in the highest dose group a lower water intake occurred in the 17th and 21st days while afterwards water intake increased, demonstrating a similar profile to food intake (Figure 15B). As it is impossible to know the food and water consumption of each animal, all consumptions were normalized to their weights as proportions, assuming that all animals had intake rates proportional to their current weights.

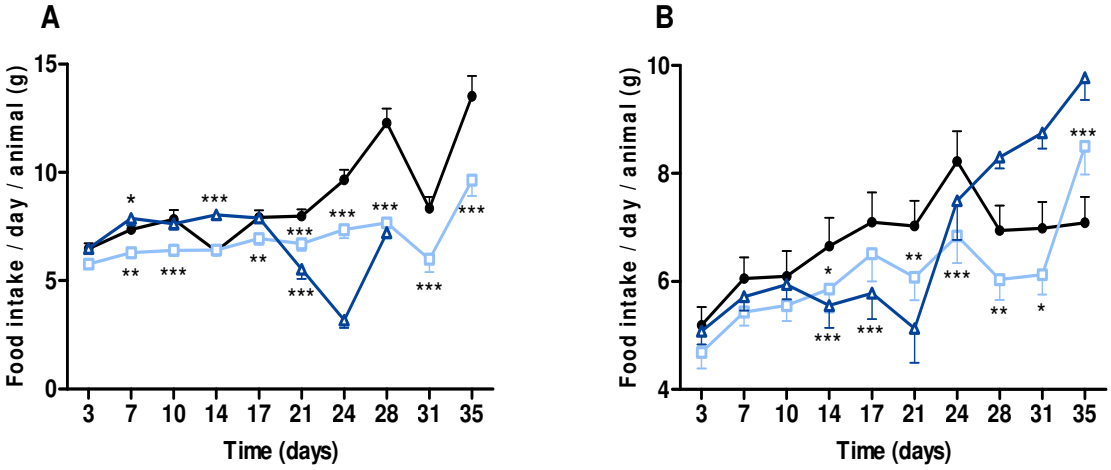

Figure 14 - Food consumption in MTX-treated (exposed to 4.5 mg/kg and 9.0 mg/kg cumulative dose) and control mice, in adult (A) and pediatric (B). Results in g/day/animal are presented as means ± standard deviation (SD), from six animals in each group up to day 21. Light blue open squares (□) represent cumulative dose of 4.5 mg/kg MTX, dark blue triangles (Δ) represent cumulative dose of 9.0 mg/kg MTX and black solid circles (●) represent saline-control. Statistical comparisons were made using two-way ANOVA followed by the Bonferroni *post hoc* test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, treatment vs. control).

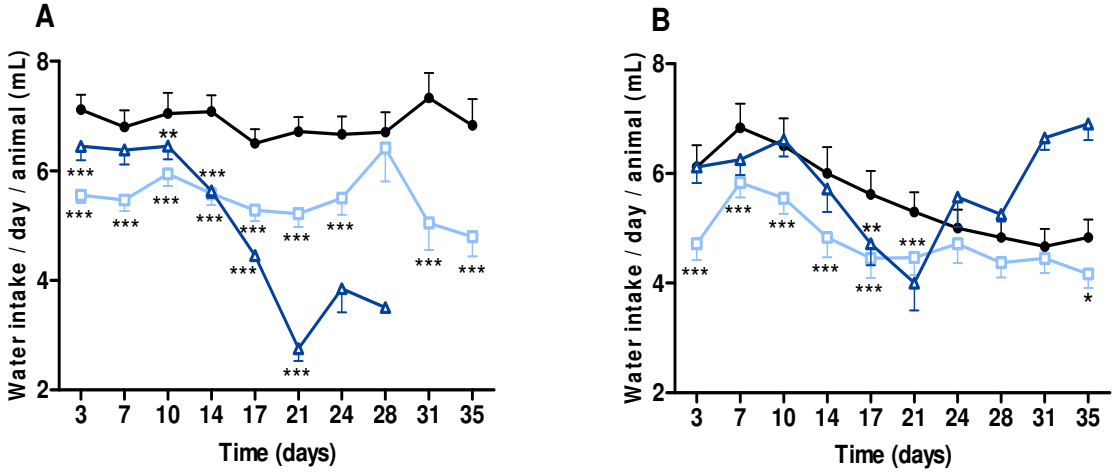

Figure 15 - Water consumption in MTX-treated (exposed to 4.5 mg/kg and 9.0 mg/kg cumulative dose) and control mice, in adult (A) and pediatric (B). Results in mL/day/animal are presented as means ± standard deviation (SD), from six animals in each group up to day 21. Light blue open squares (□) represent cumulative dose of 4.5 mg/kg MTX, dark blue triangles (Δ) represent cumulative dose of 9.0 mg/kg MTX and black solid circles (●) represent saline-control. Statistical comparisons were made using two-way ANOVA followed by the Bonferroni *post hoc* test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, treatment vs. control).

4.1.2. Plasma AST, ALT and CK-MB levels, and heart weight / body weight and liver weight / body weight ratios

Plasma levels of AST and ALT were altered in some groups. AST and ALT levels were significantly decreased in 4.5 mg/kg MTX-treated adult mice compared to levels in the control

mice (Figure 16A and C). In the pediatric population, AST levels were significantly elevated in 9.0 mg/kg MTX-treated animals, compared to the levels in the control mice (Figure 16B and D). The AST/ALT ratio is significantly increased in 9.0 mg/kg MTX of the surviving pediatric group (Figure 16F).

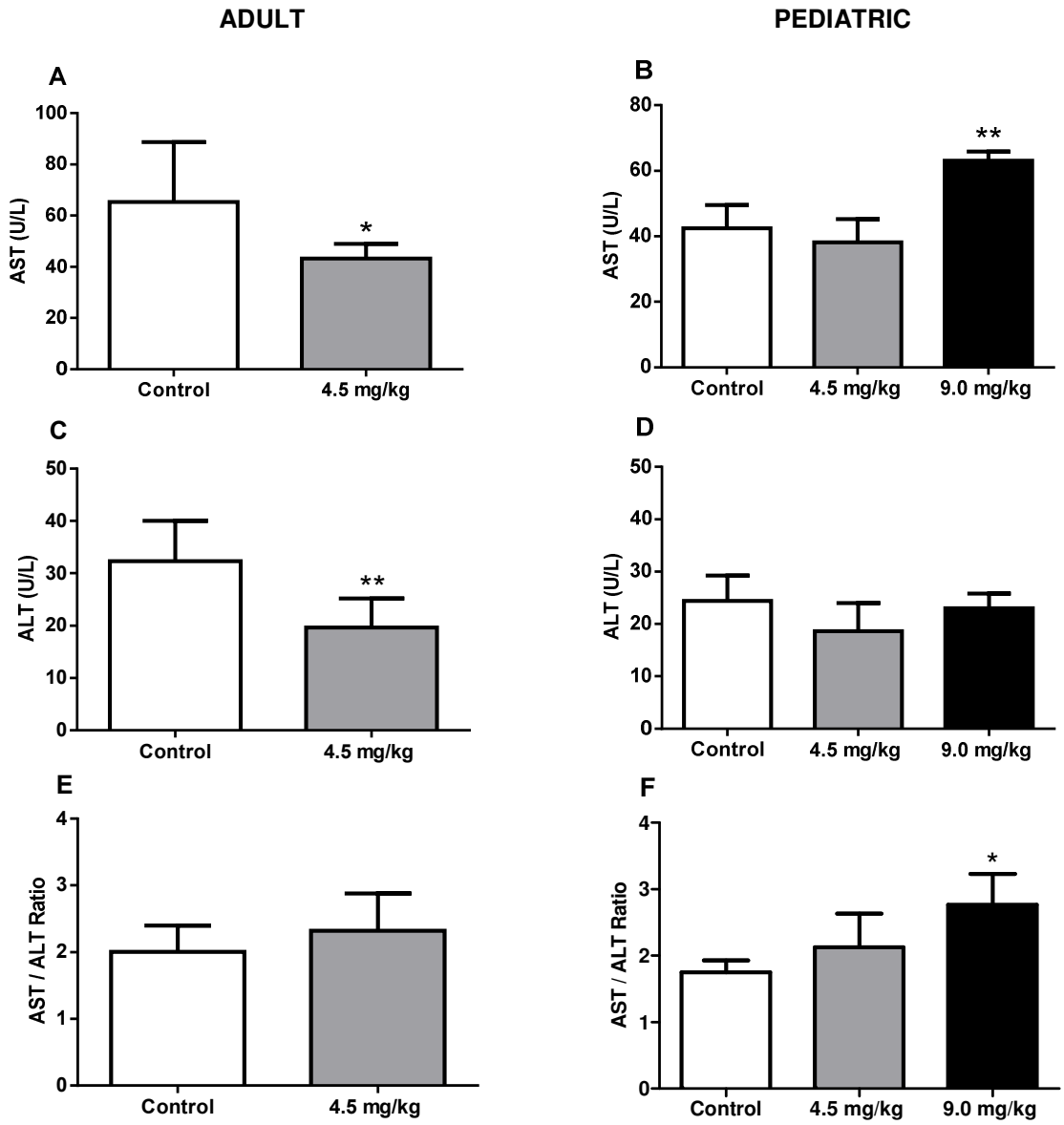

Figure 16 - Plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and its ratio in mice exposed to cumulative dose of 4.5 mg/kg MTX and 9.0 mg/kg MTX. Results, in units per liter (U/L), are presented as means ± standard deviation (SD). The number of animals varied between 2 to 6. (A and B) Plasma AST levels after MTX administration in adult and pediatric mice, respectively. Statistical comparisons were made using the t-test when two groups were considered and One-Way ANOVA, followed by the Student-Newman-Keuls *post hoc* test, when three groups were considered (*$p < 0.05$ and **$p < 0.01$, treatment vs. control). (C and D) Plasma ALT levels after MTX administration in adult and pediatric mice, respectively. Statistical comparisons were made using the t-test when two groups were considered and Kruskal-Wallis ANOVA on Ranks when three groups were considered. (E and F) AST/ALT ratio in adult and pediatric mice, respectively. Statistical comparisons were made using the t-test when two groups were considered and One-Way ANOVA, followed by the Student-Newman-Keuls *post hoc* test, when three groups were considered (*$p < 0.05$ treatment vs. control).

No significant differences were observed in CK-MB levels between MTX-treated and control mice, as well as in the percentage of heart weight / heart weight ratio of the animals in this experiment in both treatment groups. The percentage of liver weight / body weight ratio was higher in 9.0 mg/kg MTX-treated pediatric mice, showing higher liver mass in that group (Table 4).

Table 4 - Plasma creatine kinase-MB (CK-MB) levels, heart weight / body weight ratio, and liver weight / body weight ratio of the MTX-treated and control mice.

		ADULT	
Parameter	**Control**	**4.5 mg/kg**	**9.0 mg/kg**
CK-MB (U/L)	98 ± 45	89 ± 37	-
Heart weight / body weight ratio (%)	0.52 ± 0.08	0.49 ± 0.04	-
Liver weight / body weight ratio (%)	4.84 ± 0.60	5.06 ± 0.50	

		PEDIATRIC	
Parameter	**Control**	**4.5 mg/kg**	**9.0 mg/kg**
CK-MB (U/L)	97 ± 32	118 ± 72	104 ± 11
Heart weight / body weight ratio (%)	0.52 ± 0.04	0.50 ± 0.08	0.53 ± 0.01
Liver weight / body weight ratio (%)	5.38 ± 0.55	4.86 ± 0.37	6.46 ± 0.20*

Data are presented as means ± standard deviation (SD). The number of animals varied between 2 to 6. Statistical comparisons were made using the t-test when two groups were considered and One-Way ANOVA, followed by the Student-Newman-Keuls *post hoc* test, when three groups were considered (*$p < 0.05$ treatment vs. control).

4.1.3. GSHt and GSSG cellular levels in heart, liver, and kidneys

The levels of GSHt and GSSG in the heart and kidneys in 4.5 mg/kg MTX and 9.0 mg/kg MTX-treated animals can be observed in Table 5. There are no significant absolute differences in the two organs between the MTX-treated groups and controls expressed by nmol / mg protein. However, the MTX triggered alterations in redox status as seen in the GSH/GSSG ratio (Figure 17). In the heart, the pediatrics that received the cumulative dose of 9.0 mg/kg MTX had significant increased GSH/GSSH ratio when compared to control.

Table 5 - Total glutathione (GSHt) and oxidized glutathione (GSSG) cellular levels in the heart and GSHt in the kidneys, in MTX-treated (cumulative dose of 4.5 mg/kg and 9.0 mg/kg) and control mice, in adult and pediatric populations.

HEART				
	(nmol / mg protein)	**Control**	**4.5 mg/kg**	**9.0 mg/kg**
Adults	**GSHt**	5.40 ± 1.75	4.89 ± 0.48	-
	GSSG	0.38 ± 0.13	0.35 ± 0.13	-
Pediatrics	**GSHt**	6.41 ± 0.64	6.48 ± 0.64	8.75 ± 0.37
	GSSG	0.42 ± 0.10	0.34 ± 0.08	0.31 ± 0.11

KIDNEYS				
	(nmol / mg protein)	**Control**	**4.5 mg/kg**	**9.0 mg/kg**
Adults	**GSHt**	3.07 ± 1.04	4.28 ± 2.45	-
Pediatrics	**GSHt**	2.67 ± 0.58	3.32 ± 1.67	1.97 ± 0.69

Data of GSHt and GSSG levels, in nanomol per mg of protein (nmol / mg protein), are presented as means ± standard deviation (SD), and were obtained from 2-6 animals from each treatment group. Statistical comparisons were made using the t-test when two groups were considered and Kruskal-Wallis ANOVA on Ranks, when three groups were considered.

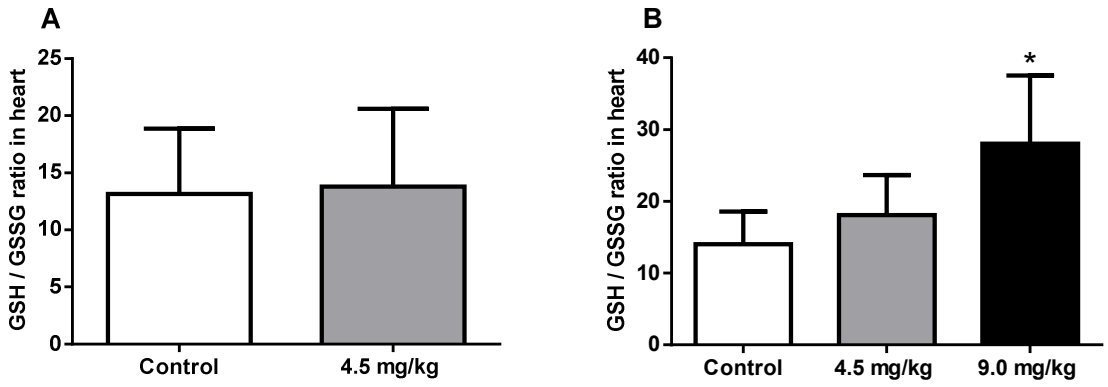

Figure 17 - (A and B) GSH / GSSG ratio in heart after MTX administration in adult and pediatric mice, respectively. Results are presented as means ± standard deviation (SD), and were obtained from 2-6 animals from each treatment group. Statistical comparisons were made using the t-test when two groups were considered and One-Way ANOVA, followed by the Student-Newman-Keuls *post hoc* test, when three groups were considered (*$p < 0.05$, treatment vs. control).

The levels of GSHt and GSSG in liver in 4.5 mg/kg MTX and 9.0 mg/kg MTX-treated animals can be observed in Table 6. There are no significant absolute differences in the liver between the MTX-treated groups and controls when expressed by nmol / mg protein. Similarly to the heart, the MTX triggers alterations in redox status as seen in the GSH/GSSG ratio (Figure 18). Oppositely to heart, in the liver, the pediatric group had significant decreased GSH/GSSG, whereas the 4.5 mg/kg MTX-treated adults group showed a significant higher ratio than controls.

Table 6 - Total glutathione (GSHt) and GSSG cellular levels in liver, in MTX-treated (cumulative dose of 4.5 mg/kg and 9.0 mg/kg) and control mice, in adult and pediatric populations.

LIVER				
	(nmol / mg protein)	**Control**	**4.5 mg/kg**	**9.0 mg/kg**
Adults	**GSHt**	43.60 ± 14.10	40.63 ± 8.83	-
	GSSG	1.42 ± 0.53	1.12 ± 0.22	-
Pediatrics	**GSHt**	48.23 ± 11.27	45.79 ± 8.39	47.28 ± 9.38
	GSSG	1.16 ± 0.30	1.37 ± 0.31	2.64 ± 1.18

Data of GSHt and GSSG levels, in nanomol per mg of protein (nmol / mg protein), are presented as means ± standard deviation (SD), and were obtained from 2-6 animals from each treatment group. Statistical comparisons were made using the t-test when two groups were considered and Kruskal-Wallis ANOVA on Ranks, when three groups were considered.

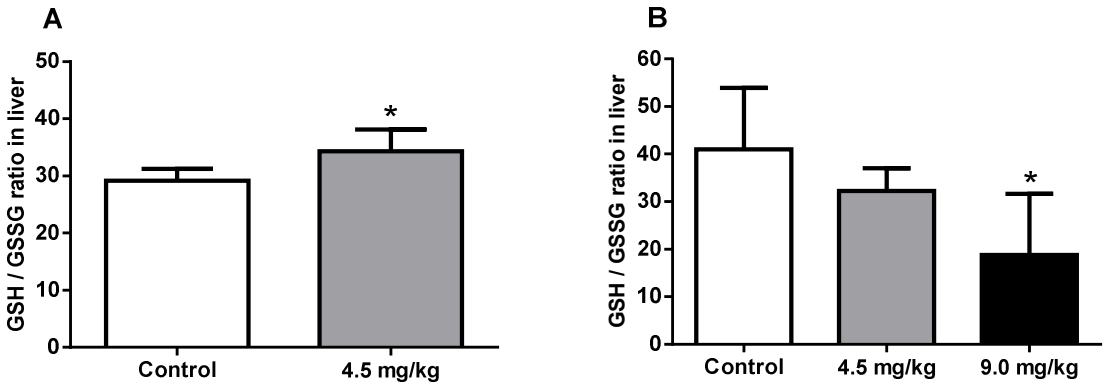

Figure 18 - (A and B) GSH/GSSG ratio in the liver after MTX administration in adult and pediatric mice, respectively. Results are presented as means ± standard deviation (SD), and were obtained from 2-6 animals from each treatment group. Statistical comparisons were made using the t-test when two groups were considered and Kruskal-Wallis ANOVA on Ranks, followed by the Dunn's *post hoc* test, when three groups were considered (*$p < 0.05$, treatment vs. control).

4.1.4. Lipid peroxidation levels

MDA can be unbound (free MDA) or bound (total MDA) to proteins and other matrix molecules. In this work, results of lipid peroxidation represent free equivalents of MDA. MTX administration in animals did not induce significant changes in MDA (indicator of lipid peroxidation) in liver and kidneys (Table 7). No measurable levels of MDA were observed in the heart. In this experiment, the MDA levels were measured just by fluorescence reading in microplate reader.

Table 7 - Free equivalents of malondialdehyde (MDA) levels in liver and kidneys in MTX-treated (cumulative dose of 4.5 mg/kg and 9.0 mg/kg) and control mice, in adult and pediatric populations.

	LIVER		
(nmol / g protein)	**Control**	**4.5 mg/kg**	**9.0 mg/kg**
Adults	27.60 ± 4.15	22.45 ± 2.54	-
Pediatrics	34.37 ± 8.15	41.08 ± 3.14	30.28 ± 6.60

	KIDNEYS		
(nmol / g protein)	**Control**	**4.5 mg/kg**	**9.0 mg/kg**
Adults	39.52 ± 6.08	51.94 ± 15.18	-
Pediatrics	43.04 ± 17.68	45.76 ± 13.19	48.91 ± 15.69

Data of MDA levels, in nanomol per g of protein (nmol / g protein) are presented as means ± standard deviation (SD), and were obtained from 2-6 animals from each treatment group. Statistical comparisons were made using the t-test when two groups were considered and One-Way ANOVA when three groups were considered.

4.2. Experiment 2

4.2.1. Body weight and daily food / water consumption

As in experiment 1 the survival percentage in the groups that received the highest cumulative dose of 9.0 mg/kg MTX was reduced (0% for adults and 33% for pediatrics), this dose was maintained but animals were sacrificed 24h after the last administration to avoid animal suffering, loss of biological sample, and assess whether MTX-induced damage had already occurred in an early stage. Additionally, instead of the cumulative dose of 4.5 mg/kg MTX administration, the mice received a cumulative dose of 7.0 mg/kg MTX twice a week for three weeks, and were maintained in a drug-free period for developmental of cumulative toxicity. However, to maintain the survival percentage at 100%, the animals of dose 7.0 mg/kg MTX were not sacrificed in 37th day of the experiment as in the experiment 1: the animals showed signs of toxicity as in experiment 1 and in order to minimize the suffering, humane endpoints were taken into account and the animals were sacrificed one week earlier (day 31).

In the adult population, the average body weight was different in the 7.0 mg/kg MTX and 9.0 mg/kg MTX groups (Figure 19A and C). The 9.0 mg/kg MTX-treated mice had similar average body weight compared to their controls during the course of the experiment, whereas the 7.0 mg/kg MTX-treated mice had lower average body weight than their controls after reaching the maximum cumulative dose. Significant differences in the gain body weight occurred after the 21st day, and loss of weight in the 24th day, in this group. In the pediatric

population, the 7.0 mg/kg MTX-treated animals had significant less body weight gain compared to controls after the 17th day. The 9.0 mg/kg MTX-treated animals in the last evaluation also showed significant loss of body weight (Figure 19B and D).

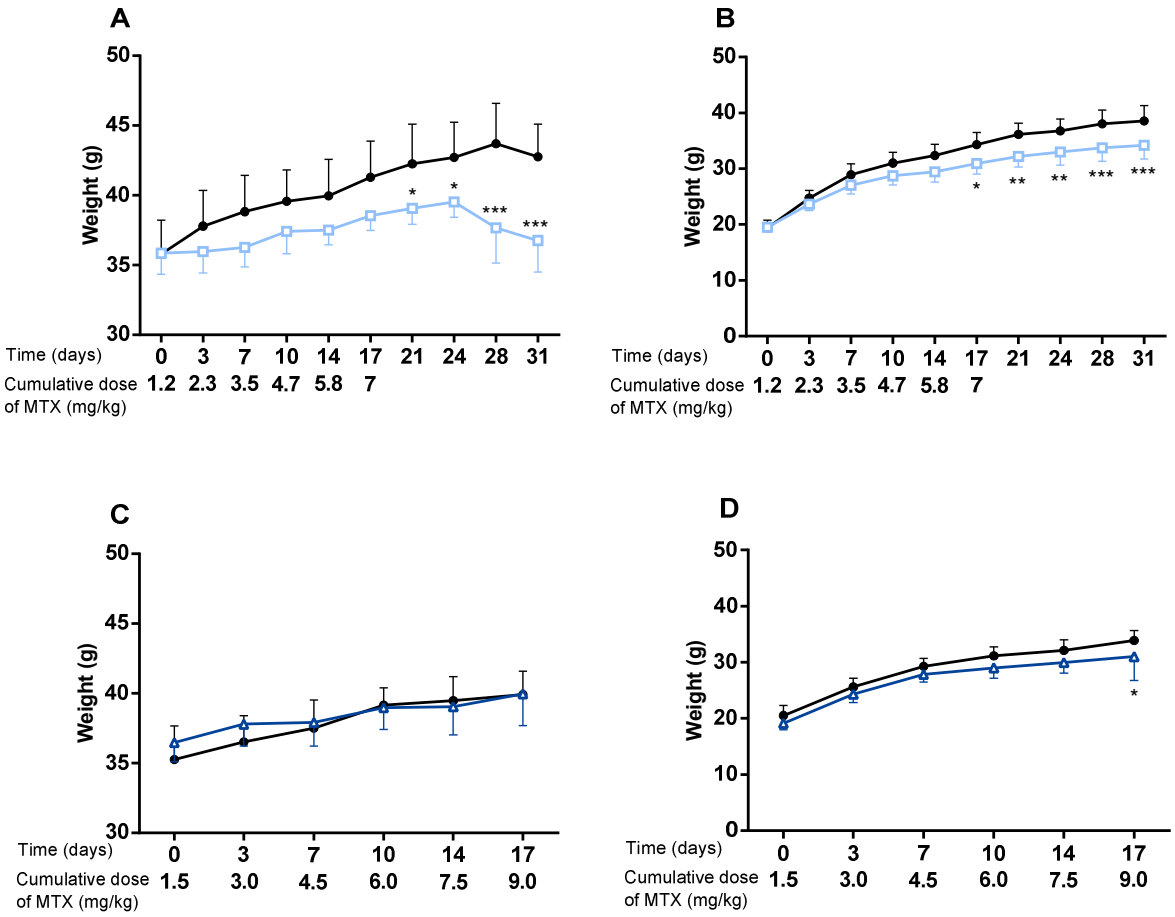

Figure 19 - (A and B) Average body weight in 7.0 mg/kg MTX-treated adult and pediatric mice, respectively. (C and D) Average body weight in 9.0 mg/kg MTX-treated adult and pediatric mice, respectively. Results in grams (g) are presented as mean ± standard deviation (SD), from eight animals in each group, excepting in 7.0 mg/kg MTX-treated pediatrics (n = 7). Light blue open squares (□) represent cumulative dose of 7.0 mg/kg MTX treatment, dark blue triangles (Δ) represent cumulative dose of 9.0 mg/kg MTX treatment and black solid circles (●) represent saline-control treatment. Statistical comparisons were made using two-way ANOVA followed by the Bonferroni *post hoc* test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, treatment vs. control).

Differences in food consumption were more significant in the case of the 7.0 mg/kg MTX-treated pediatrics (Figure 20B), with significant lower consumption from 14th day until the end of the experiment, compared to the control group. The adults that received the same dose had significant different food consumption only in the week following the last MTX administration, with a higher consumption compared to the control group (Figure 20A). The adult animals that received a cumulative dose of 9.0 mg/kg MTX had two different situations regarding their food consumption: significant lower consumption at the beginning of the

experiment and higher at the end, compared to control (Figure 20C). The pediatric mice, in a general manner, had lower consumption during all the period of the experiment, compared to control in both concentrations (Figure 20B and D), evidencing a similar consumption behavior with the 7.0 mg/kg MTX-treated pediatrics.

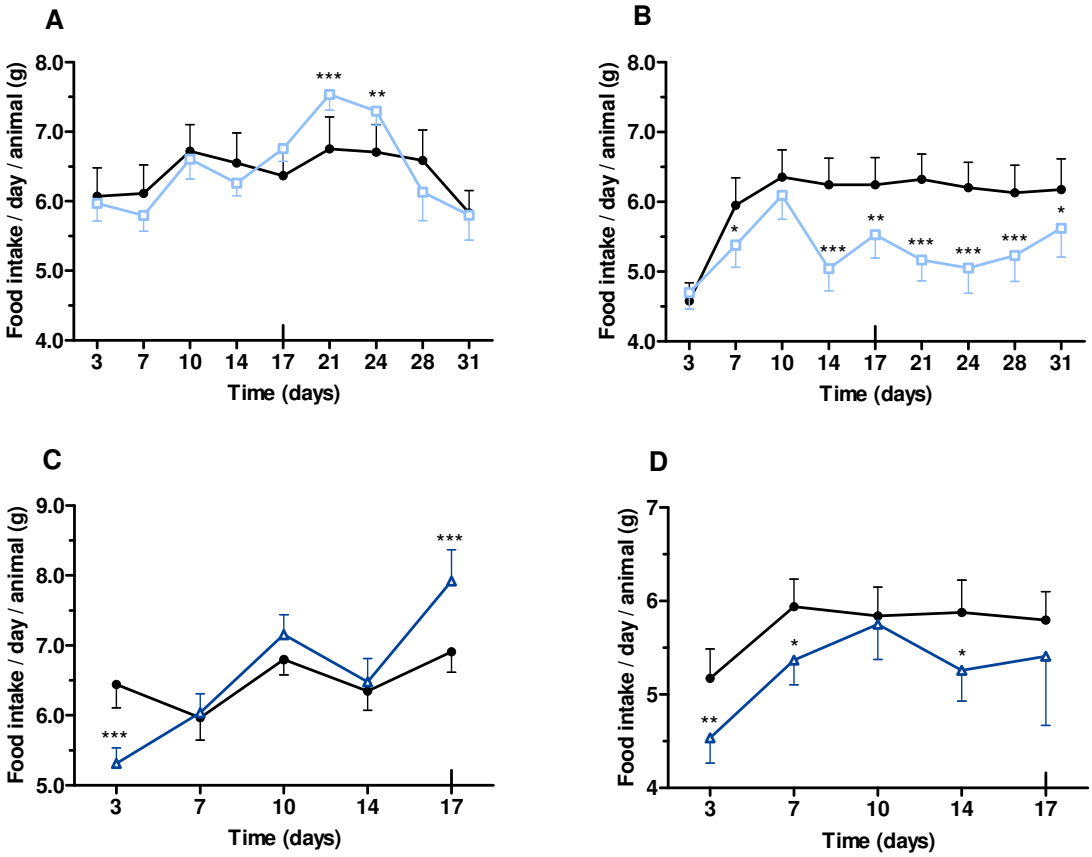

Figure 20 - (A and B) Food consumption in 7.0 mg/kg MTX-treated adult and pediatric mice, respectively. (C and D) Food consumption in 9.0 mg/kg MTX-treated adults and pediatrics, respectively. Results in grams (g) are presented as mean ± standard deviation (SD), from eight animals in each group, excepting 7.0 mg/kg MTX-treated pediatrics (n = 7). Light blue open squares (□) represent cumulative dose of 7.0 mg/kg MTX treatment, dark blue triangles (Δ) represent cumulative dose of 9.0 mg/kg MTX treatment and black solid circles (●) represent saline-control treatment. Small vertical line in 17th day (x axis) indicates the last MTX administration. Statistical comparisons were made using two-way ANOVA followed by the Bonferroni *post hoc* test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, treatment vs. control).

Data of water consumption are presented in Figure 21 and a significant lower consumption in all groups is observed, excepting in 9.0 mg/kg MTX-treated pediatric mice, compared to respective control groups. In the adults, before reaching the total cumulative dose, the water intake was significantly impaired. In the 7.0 mg/kg-treated pediatric mice, only in the last administrations and after, changes were seen.

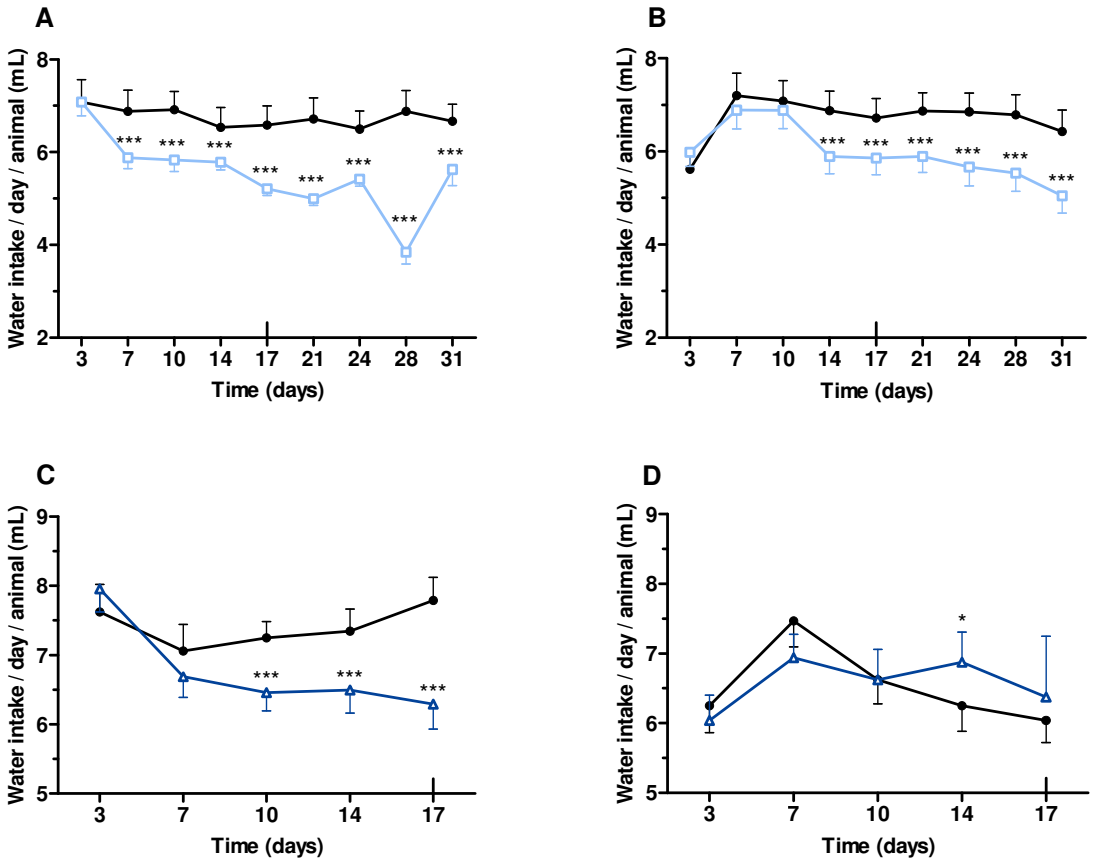

Figure 21 - (A and B) Water consumption in 7.0 mg/kg MTX-treated adult and pediatric mice, respectively. (C and D). Water consumption in 9.0 mg/kg MTX-treated adults and pediatrics, respectively. Results in grams mL/day/animal are presented as mean ± standard deviation (SD), from eight animals in each group, excepting 7.0 mg/kg MTX-treated pediatrics (n = 7). Light blue open squares (□) represent cumulative dose of 7.0 mg/kg MTX treatment, dark blue triangles (Δ) represent cumulative dose of 9.0 mg/kg MTX treatment and black solid circles (●) represent saline-control treatment. Small vertical line in 17th day (x axis) indicates the last MTX administration. Statistical comparisons were made using two-way ANOVA followed by the Bonferroni *post hoc* test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, treatment vs. control).

4.2.2. Ratios of heart weight / body weight and liver weight / body weight

In table 8, the percentage of ratios of heart weight / body weight can be observed. The cumulative dose of 7.0 mg/kg MTX induced a significant difference in the ratio in the adults group, which presented a decreased heart weight compared to control. In this same dose group, MTX induced a significant decrease in the percentage of liver weight / body weight ratio compared to control (Table 9). No other significant differences were observed for these parameters.

Table 8 - Ratio of heart weight / body weight of the MTX-treated and control mice.

	Heart weight / body weight ratio (%)			
	Control 7.0 mg/kg	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
Adult	0.47 ± 0.04	0.42 ± 0.03 *	0.52 ± 0.03	0.50 ± 0.11
Pediatric	0.46 ± 0.04	0.46 ± 0.07	0.50 ± 0.05	0.51 ± 0.08

Results are presented as means ± standard deviation (SD) from 7-8 animals of each group. Statistical comparisons were made using the t-test (*$p < 0.05$, treatment vs. control).

Table 9 - Ratio of liver weight / body weight ratio of the MTX-treated and control mice.

	Liver weight / body weight ratio (%)			
	Control 7.0 mg/kg	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
Adult	5.41 ± 0.38	4.19 ± 0.91 **	5.65 ± 0.52	4.96 ± 0.76
Pediatric	5.87 ± 0.30	5.58 ± 0.41	6.44 ± 0.57	6.05 ± 0.80

Results are presented as means ± standard deviation (SD) from 7-8 animals of each group. Statistical comparisons were made using the t-test (**$p < 0.01$, treatment vs. control).

4.2.3. Lymphocytes determination

Changes in the levels of lymphocytes in MTX-treated mice are presented in Table 10. Since data are from a small number of animals, no statistical comparisons were done. The levels of lymphocytes seem to be lower in animals treated with cumulative dose of 9.0 mg/kg MTX, in both populations. No apparent changes occurred in the other hematological parameters (data not shown).

Table 10 - Lymphocytes changes in MTX-treated and control mice.

	Lymphocytes (10^3 / mm^3)			
	Control 7.0 mg/kg	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
Adults	3.9 ± 2.4	1.8 ± 0.4	2.0 ± 0.2	0.8 ± 0.1
Adolescents	3.9 ± 0.0	2.9 ± 0.0	2.1 ± 0.4	0.8 ± 0.4

Data of lymphocytes levels, in 10^3 / mm^3, are present as means ± standard deviation (SD) and were obtained from 1-3 animals of each group. No statistical treatment was done due to the small number of animals tested.

4.2.4. Plasma AST and ALT levels and AST/ALT ratio

Plasma levels of AST and ALT were not altered in this experiment, in both populations and in both cumulative doses. Likewise, no differences were found in the AST/ALT ratio (Table 11).

Table 11 - Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels and AST/ALT ratio of the MTX-treated and control mice.

		ADULT		
Parameter	**Control 7.0 mg/kg**	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
AST (U/L)	65.75 ± 21.79	87.13 ± 32.46	49.25 ± 10.14	61.00 ± 21.35
ALT (U/L)	39.63 ± 22.75	46.00 ± 37.04	31.25 ± 3.85	39.63 ± 29.59
AST/ALT Ratio	1.80 ± 0.42	2.46 ± 0.91	1.58 ± 0.26	1.93 ± 0.77
		PEDIATRIC		
Parameter	**Control 7.0 mg/kg**	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
AST (U/L)	51.33 ± 8.31	70.20 ± 17.92	39.14 ± 4.18	44.43 ± 14.62
ALT (U/L)	37.00 ± 15.66	26.75 ± 3.30	27.29 ± 5.77	29.29 ± 13.07
AST/ALT Ratio	1.69 ± 0.54	2.49 ± 0.40	1.52 ± 0.43	1.68 ± 0.71

Data of AST and ALT levels, in units per liter (U/L), are presented as means ± standard deviation (SD) and were from 4-8 animals of each group. Statistical comparisons were made using the Mann-Whitney Rank Sum test between treated groups and the respective controls.

4.2.5. Total-CK and CK-MB levels

Plasma levels of total-CK were quantified in this experiment and are depicted in Table 12. There were no alterations in this parameter in both populations and in both treatment doses.

Table 12 - Total CK (creatine kinase) in plasma of MTX-treated and control mice.

	Total-CK (U/L)			
	Control 7.0 mg/kg	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
Adult	78.38 ± 25.01	192.00 ± 141.93	114.25 ± 57.77	120.88 ± 64.62
Pediatric	45.14 ± 7.67	54.75 ± 4.03	115.38 ± 62.80	89.00 ± 39.90

Data of total-CK, in units per liter (U/L), are present as means ± standard deviation (SD) and were from 4-8 animals of each group. Statistical comparisons were made using the Mann-Whitney Rank Sum test between treated groups and the respective controls.

The data concerning the plasma CK-MB levels in MTX-treated and control mice are shown in Figure 22. MTX-treated adults did not present any changes in their plasma CK-MB values compared to controls. In the pediatric mice, the plasma CK-MB results were significant higher in 7.0 mg/kg MTX-treated mice and significant lower in 9.0 mg/kg MTX-treated mice, compared to respective controls.

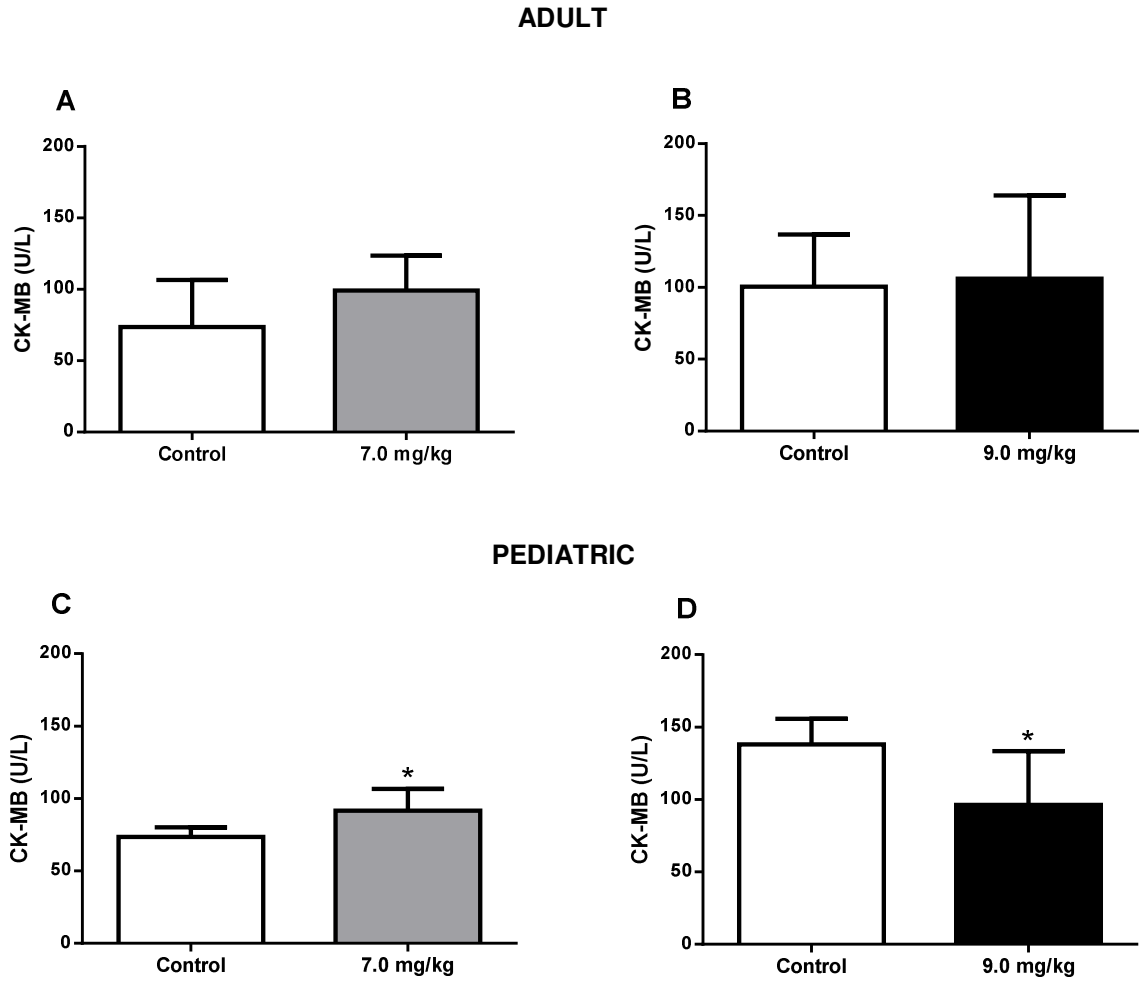

Figure 22 - Plasma levels of CK-MB in 7.0 mg/kg and 9.0 mg/kg MTX-treated animals. (A and B) CK-MB levels in plasma of adults after cumulative administration of 7.0 mg/kg MTX and 9.0 mg/kg MTX, respectively. (C and D) CK-MB levels in plasma of pediatrics after administration of 7.0 mg/kg MTX and 9.0 mg/kg MTX, respectively. Results, in units per liter (U/L), are presented as means ± standard deviation (SD), and were obtained from 5-8 animals from each treatment group. Statistical comparisons were made using the Mann-Whitney Rank Sum test (*$p < 0.05$, treatment vs. control).

4.2.6. GSHt and GSSG cellular levels in heart, liver and kidneys

In Figure 23, the levels of GSHt and GSSG in heart of 7.0 mg/kg MTX-treated adult mice (A and B, respectively) and pediatric mice (C and D, respectively) can be observed. Differences were found in the GSHt levels of the pediatric population, presenting significant higher values when compared to controls. No significant absolute differences in other glutathione contents between the others groups were found.

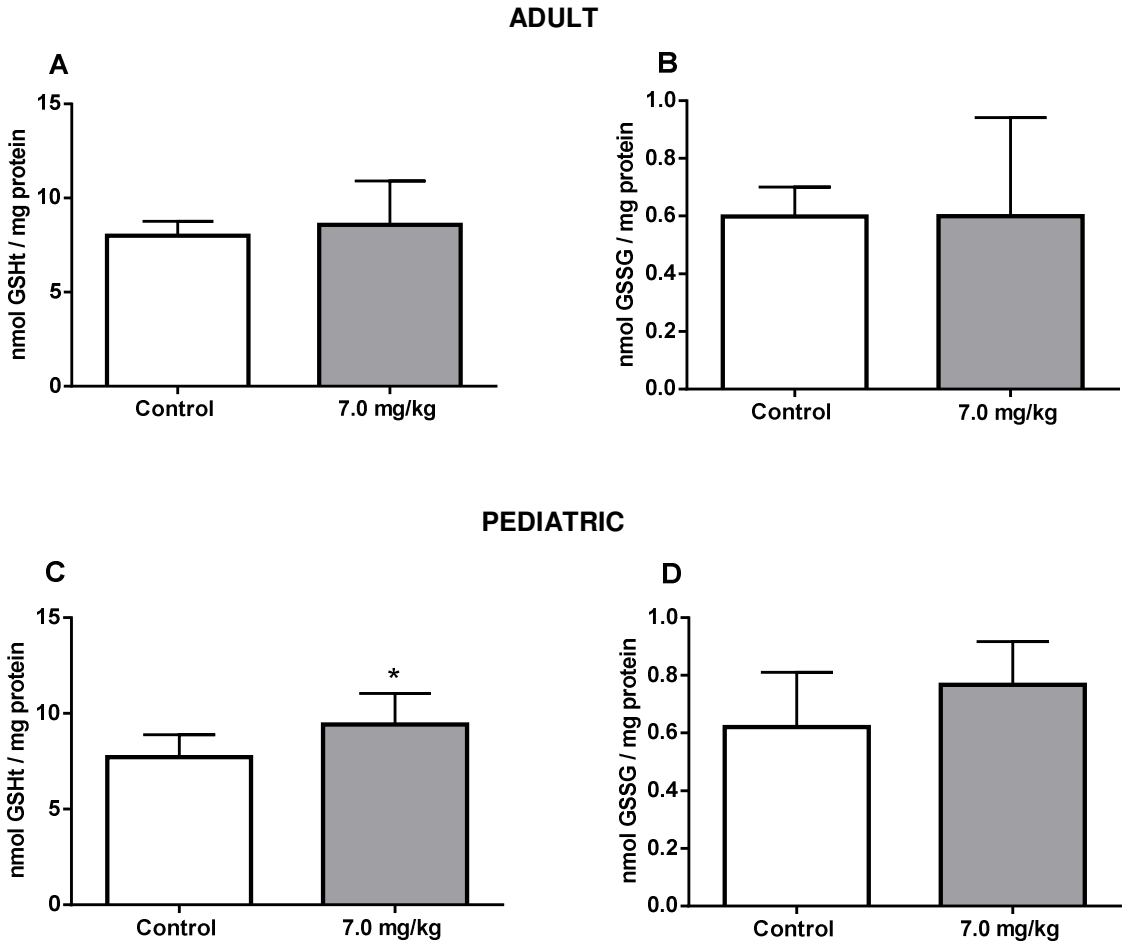

Figure 23 - (A and B) GSHt and GSSG levels in the heart, respectively, after cumulative 7.0 mg/kg MTX administration in adult mice. (C and D) GSHt and GSSG levels in the heart, respectively, after cumulative 7.0 mg/kg MTX administration in pediatric mice. Results are presented as means ± standard deviation (SD), and were obtained from 7-8 animals from each treatment. Statistical comparisons were made using the Mann-Whitney Rank Sum test between the treated group and respective control (*$p < 0.05$, treatment vs. control).

The GSHt and GSSG values of the 9.0 mg/kg-treated adults (A and B, respectively) and pediatrics (C and D, respectively) mice are depicted in Figure 24. Higher GSSG levels were found in the 9.0 mg/kg MTX-treated adult animals, compared to controls. No significant absolute differences between the others groups were found.

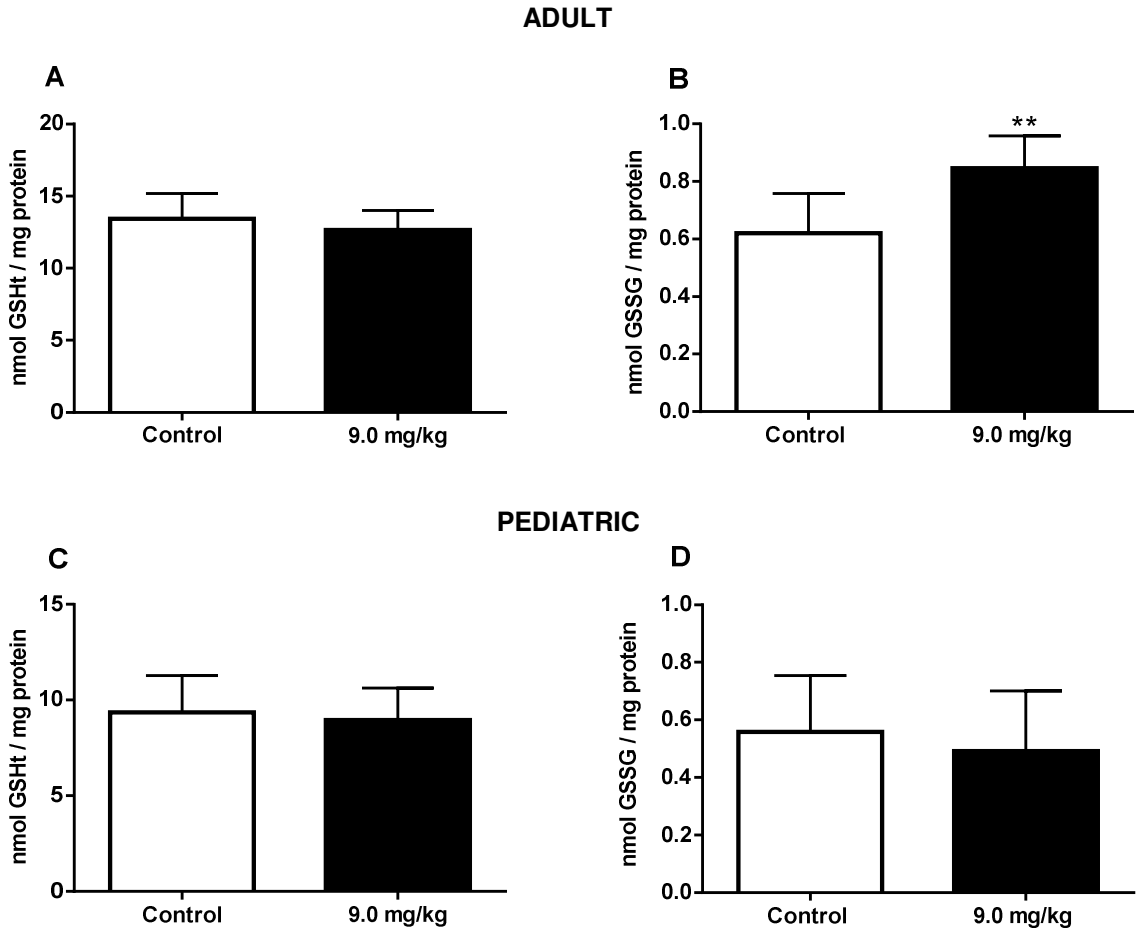

Figure 24 - (A and B) GSHt and GSSG levels in the heart, respectively, after cumulative 9.0 mg/kg MTX administration in adult mice. (C and D) GSHt and GSSG levels in the heart, respectively, after cumulative 9.0 mg/kg MTX administration in pediatric mice. Results are presented as means ± standard deviation (SD), and were obtained from 7-8 animals from each treatment. Statistical comparisons were made using the Mann-Whitney Rank Sum test between the treated group and respective control (**$p < 0.05$, treatment vs. control).

The levels of GSHt and GSSG in liver and kidneys in 7.0 mg/kg MTX and 9.0 mg/kg MTX-treated animals can be observed in Table 13. The liver of the 7.0 mg/kg MTX-treated adults showed significant decreased GSHt levels. The kidneys of the 9.0 mg/kg MTX-treated pediatrics also presented altered values, with significant decrease in GSSG levels. No other differences were found in these organs in the other treatment groups, as well as no differences in the GSH/GSSG ratios (data not shown).

Table 13 - Total glutathione (GSHt) and GSSG cellular levels in liver and kidneys in MTX-treated (cumulative dose of 7.0 mg/kg and 9.0 mg/kg) and control mice, in adult and pediatric populations.

		LIVER			
	(nmol / mg protein)	**Control 7.0 mg/kg**	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
Adults	**GSHt**	58.35 ± 8.49	40.92 ± 15.74 *	56.56 ± 16.82	54.29 ± 11.06
	GSSG	23.45 ± 6.48	19.14 ± 6.89	21.90 ± 6.20	19.29 ± 2.59
Pediatrics	**GSHt**	46.74 ± 9.00	47.45 ± 12.82	45.45 ± 6.39	47.65 ± 16.12
	GSSG	18.69 ± 6.35	20.16 ± 9.12	10.39 ± 3.43	10.67 ± 2.63

		KIDNEYS			
	(nmol / mg protein)	**Control 7.0 mg/kg**	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
Adult	**GSHt**	1.15 ± 0.17	1.29 ± 0.45	0.74 ± 0.24	0.84 ± 0.20
	GSSG	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Pediatric	**GSHt**	1.14 ± 0.40	1.17 ± 0.40	1.03 ± 0.14	1.21 ± 0.27
	GSSG	0.02 ± 0.02	0.02 ± 0.02	0.03 ± 0.01	0.01 ± 0.01 **

Data of GSHt and GSSG levels, in nanomol per mg of protein (nmol / mg protein), are presented as means ± standard deviation (SD), and were obtained from 7-8 animals from each treatment group. Statistical comparisons were made using the t-test for hepatic GSHt and GSSG content and the Mann-Whitney Rank Sum test for renal GSHt and GSSG content (*$p < 0.05$, **$p < 0.01$, treatment vs. control).

4.2.7. Lipid peroxidation levels

The main problem of the lipid peroxidation assessment using the TBARS method is its lack of sensitivity and specificity (Grotto et al., 2009), as a result of the presence of interfering agents both in the colorimetric and fluorescence measurements. Although lipid peroxidation was first assessed in a fluorescence plate reader (data not shown), in the experiment 2 it was complemented by the measurement of MDA equivalents by a HPLC method (Ying et al., 2008). This later method is more accurate for the detection of lipid peroxidation products (Grotto et al., 2009). The isocratic elution and separation of the $MDA(TBA)_2$ adduct from other TBA adducts in biological samples was accomplished. The retention time for the $MDA(TBA)_2$ adduct was nearly 4.8 min and the 532 nm wavelength was selected according to the literature for integrating areas of $MDA(TBA)_2$ adduct (Ying et al., 2008). The absorption spectrum (500-600 nm) of the $MDA(TBA)_2$ adduct is depicted in Figure 25. Representative chromatograms obtained from a 7.0 mg/kg MTX-treated adult liver sample, before and after TBA derivatization, are shown in Figure 26A (dashed and continuous line, respectively), as well as a chromatogram from a 6 µM MDA standard after TBA derivatization (Figure 26B).

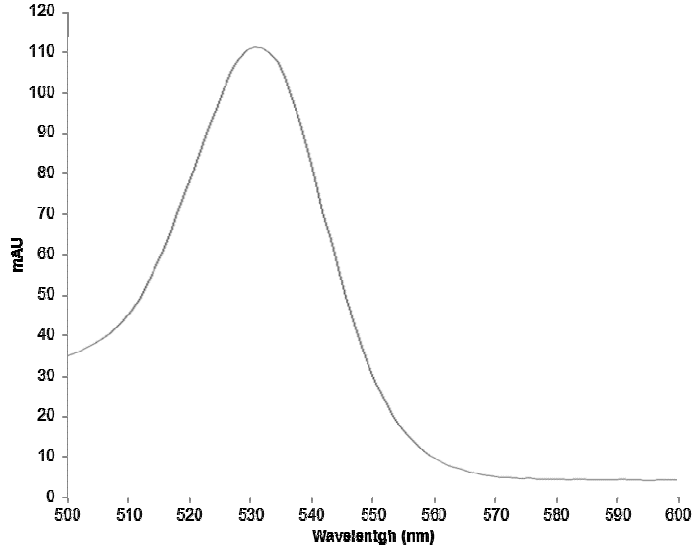

Figure 25 - The spectrum of MDA(TBA)$_2$ adduct from 500 to 600 nm (6 µM MDA standard).

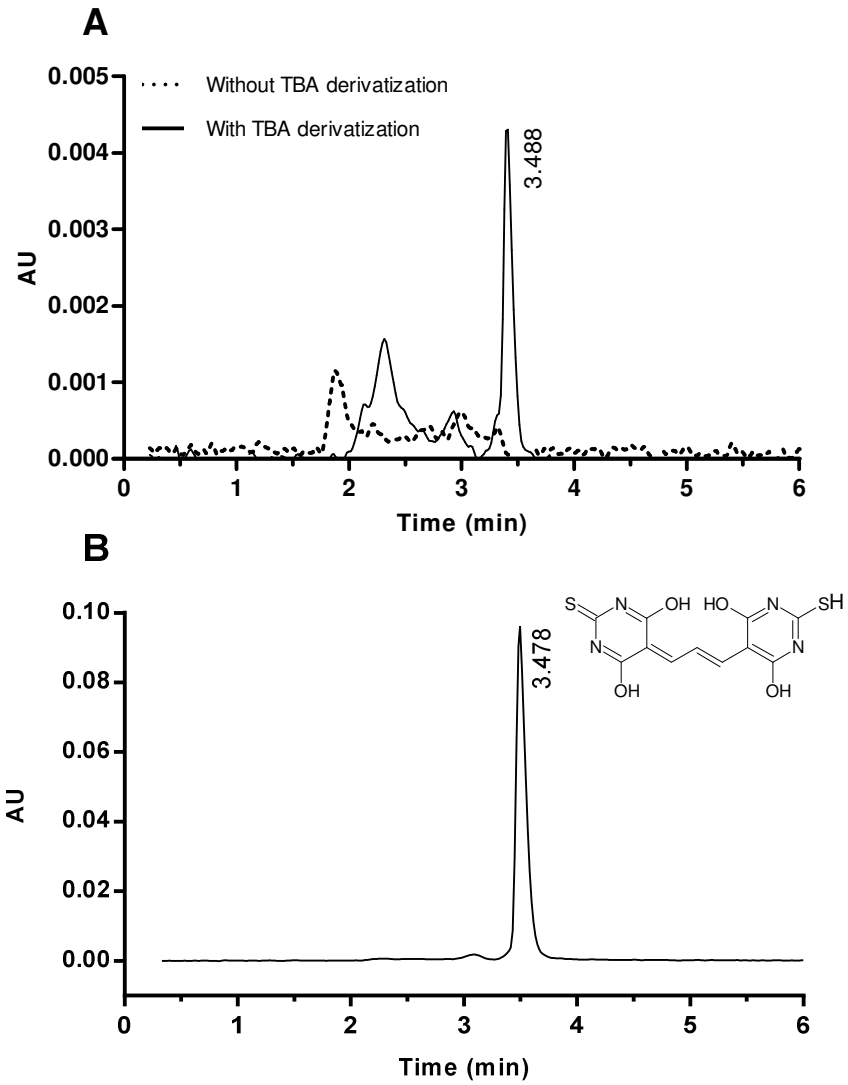

Figure 26 - (A) Chromatograms of a 7.0 mg/kg MTX-treated adult liver sample, before and after TBA derivatization (dashed and continuous line, respectively). (B) Chromatogram from a 6 µM MDA standard after TBA derivatization and the chemical structure of the MDA(TBA)$_2$ adduct.

No free equivalents of MDA in heart are presented in this study, since the all values were below of the lowest standard used. Regarding to the liver, in the 9.0 mg/kg MTX-treated pediatric group, lower lipid peroxidation levels were observed when compared to control. No differences were found in the liver of the 7.0 mg/kg MTX pediatric group, neither in all the adults groups. In kidneys, high standard deviations and no differences between populations were observed (Table 14).

Table 14 - Malondialdehyde (MDA) levels in liver and kidneys in MTX-treated (cumulative dose of 7.0 mg/kg and 9.0 mg/kg) and control mice, in adult and pediatric populations.

	LIVER			
nmol / g protein	**Control 7.0 mg/kg**	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
Adult	8.48 ± 4.61	6.27 ± 8.70	11.07 ± 11.47	16.24 ± 3.86
Pediatric	4.22 ± 4.13	4.76 ± 3.44	13.40 ± 3.34	7.12 ± 3.40 **

	KIDNEYS			
nmol / g protein	**Control 7.0 mg/kg**	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
Adult	61.26 ± 34.28	84.81 ± 72.33	53.97 ± 33.33	41.89 ± 26.26
Pediatric	59.93 ± 28.74	42.91 ± 15.80	41.20 ± 20.92	40.55 ± 12.51

Data of MDA levels, in nanomol per g of protein (nmol / g protein) are presented as means ± standard deviation (SD), and were obtained from 4-8 animals from each treatment group. Statistical comparisons were made using the Mann-Whitney Rank Sum test (**$p < 0.01$, treatment vs. control).

4.2.8. ATP levels

To understand if MTX has different chronic effects in cellular energetics of the two populations, intracellular ATP levels were measured in the heart, liver, and kidneys of the animals exposed to MTX. Alterations in hepatic ATP levels are evident in Figure 27. In adult mice, an approximately 3-fold and 2-fold significant decrease in ATP levels occurred, in 9.0 mg/kg MTX and 7.0 mg/kg MTX treatment doses, respectively (Figure 27A and B). In the pediatric population, changes also occurred in ATP, although in a lower extent. A significant decrease in the hepatic ATP levels occurred in 9.0 mg/kg group when compared to control (Figure 27C). In the 7.0 mg/kg MTX tested concentration, no significance changes were detected. Regarding heart and kidney, no significant differences were observed any treatment group as can be seen in Table 15.

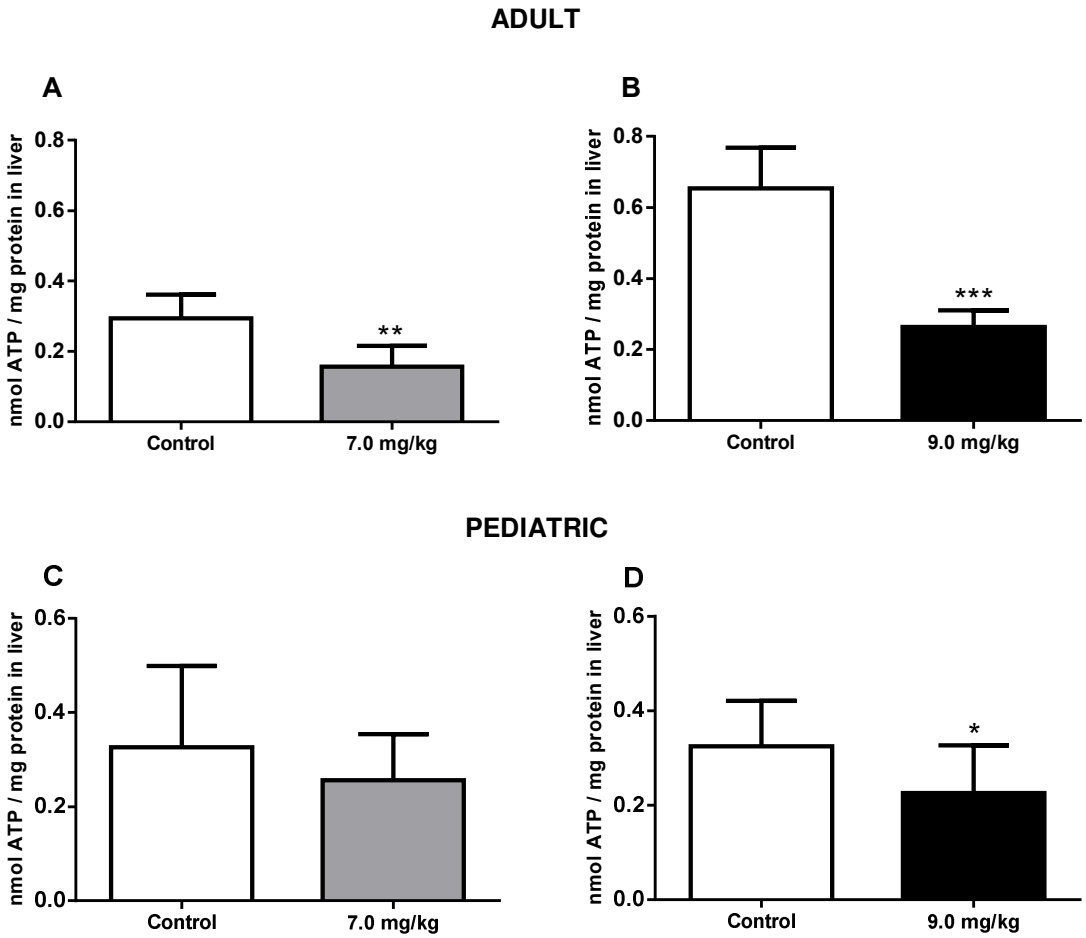

Figure 27 - ATP levels in the liver of mice exposed to cumulative dose of 7.0 mg/kg MTX and 9.0 mg/kg MTX. (A and B) ATP levels in the liver of adults after cumulative dose of 7.0 mg/kg MTX and 9.0 mg/kg MTX, respectively. (C and D) ATP levels in liver of pediatrics after cumulative dose of 7.0 mg/kg MTX and 9.0 mg/kg MTX, respectively. Results, in nmol / mg protein, are presented as means ± standard deviation (SD), and were obtained from 7-8 animals from each treatment group. Statistical comparisons were made using the Mann-Whitney Rank Sum (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ treatment vs. control).

Table 15 - ATP levels in the heart and kidneys of mice exposed to cumulative dose of 9.0 mg/kg MTX and 7.0 mg/kg MTX.

	HEART			
nmol /mg protein	**Control 7.0 mg/kg**	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
Adult	3.92 ± 1.34	3.23 ± 1.39	2.85 ± 0.95	3.33 ± 1.32
Pediatric	3.94 ± 1.97	3.77 ± 1.42	2.99 ± 1.19	2.96 ± 1.23
	KIDNEYS			
nmol /mg protein	**Control 7.0 mg/kg**	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
Adult	0.20 ± 0.02	0.20 ± 0.02	0.24 ± 0.06	0.19 ± 0.03
Pediatric	0.19 ± 0.02	0.18 ± 0.03	0.24 ± 0.03	0.28 ± 0.09

Results, in nmol per mg of protein (nmol / mg protein), are presented as means ± standard deviation (SD) and were obtained from 7-8 animals from each treatment group. Statistical comparisons were made using the Mann-Whitney Rank Sum between treated groups and the respective controls.

4.2.9. Caspase-3, -8 and -9 activities

To assess whether MTX induced cell death via apoptosis, the activities of caspase-3, -8 and -9 were measured through a method based in the cleavage of the respective caspase substrates. Caspase-3 activity was significantly decreased in pediatric mice population treated with cumulative dose of 9.0 mg/kg MTX (*$p < 0.05$) (Table 16). No other differences were observed in the activity of caspase-3 in other populations. Regarding the activity of caspase-8 and caspase-9, no significant differences were observed in either MTX-cumulative doses or populations.

Table 16 - Caspase-3, -8 and -9 activities in the heart of mice treated with total cumulative doses of 7.0 mg/kg MTX or 9.0 mg/kg MTX.

Parameter	**ADULT**			
(Fluorescent units/ mg protein)	**Control 7.0 mg/kg**	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
Caspase-9	92 ± 96	81 ± 132	153 ± 118	254 ± 118
Caspase-8	788 ± 259	747 ± 261	1605 ± 193	1697 ± 442
Caspase-3	587 ± 131	597 ± 197	1861 ± 324	2281 ± 702

Parameter	**PEDIATRIC**			
(Fluorescent units / mg protein)	**Control 7.0 mg/kg**	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
Caspase-9	115 ± 80	131 ± 116	364 ± 149	461 ± 283
Caspase-8	980 ± 300	998 ± 292	2310 ± 429	2190 ± 558
Caspase-3	685 ± 186	621 ± 155	2771 ± 200	1953 ± 563*

Data of caspase 9, 8 and 3 activities, in fluorescent units / mg protein, are presented as means ± standard deviation (SD), and were obtained from 4-8 animals from each treatment group. Statistical comparisons were made using the Mann-Whitney Rank Sum test (*$p < 0.05$, treatment vs. control).

4.2.10. Structural examination of heart

Histologic examination by light microscopy of cardiac morphology in all groups of MTX-treated mice was performed. Lesions in the cardiac tissue were microscopically characterized by cellular degeneration, interstitial inflammatory cell infiltration, and necrotic zones. Results of semi-quantitative analysis of the MTX-treated and controls groups are

presented in Table 17. Major qualitative and representative structural alterations are depicted in Figure 28.

The two cumulative doses of MTX provoked cardiac damage to both populations. In MTX-treated adults, the presence of cellular edema, cytoplasmic vacuolization of cardiomyocytes interstitial inflammatory cell infiltration, as well as some necrotic zones was evident. Similarly, pediatric group showed interstitial inflammatory cell infiltration but in a lower extent when compared to adults, showing less signs of cytoplasmic vacuolization and necrotic zones.

In 7.0 mg/kg MTX-treated animals, namely in adults, the cardiac damage is well visible in the endocardium towards to pericardium zone, while in 9.0 mg/kg MTX-treated animals the lesions appeared essentially in the endocardium. The pediatric animals of the 7.0 mg/kg MTX dose presented a well conserved periphery. In the 7.0 mg/kg MTX-treated pediatric population, the existence of sporadic cardiomyocytes showing large nuclei and more than one nucleolus was well observed, being presumably in activity. All groups showed myocardium with preserved structure (score = 0), therefore data regarding tissue disorganization were omitted from the table.

Table 17 - Semi-quantitative analysis of the morphological injury parameters of MTX-treated and controls groups, in adult and pediatric populations.

	ADULT			
	Control 7.0 mg/kg	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
Cellular degeneration	0.64 ± 0.63	1.42 ± 0.71 ****	0.50 ± 0.67	1.47 ± 0.76 ****
Necrosis	0.00 ± 0.00	0.23 ± 0.43 ***	0.00 ± 0.00	0.31 ± 0.47 ****
Inflammatory activity	0.30 ± 0.46	1.02 ± 0.49 ****	0.26 ± 0.45	0.91 ± 0.70 ****
	PEDIATRIC			
	Control 7.0 mg/kg	**7.0 mg/kg**	**Control 9.0 mg/kg**	**9.0 mg/kg**
Cellular degeneration	0.50 ± 0.54	0.82 ± 0.63 **	0.38 ± 0.49	0.98 ± 0.74 ****
Necrosis	0.00 ± 0.00	0.04 ± 0.20	0.00 ± 0.00	0.13 ± 0.34 *
Inflammatory activity	0.22 ± 0.42	0.85 ± 0.52 ****	0.25 ± 0.44	0.81 ± 0.50 ****

Results, given in scores, are presented as means ± standard deviation (SD) and were obtained from 3 animals from each treatment group, excepting the control of 7.0 mg/kg MTX-treated pediatric group (n = 2). Statistical comparisons were made using the Mann-Whitney Rank Sum (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, treatment vs. control).

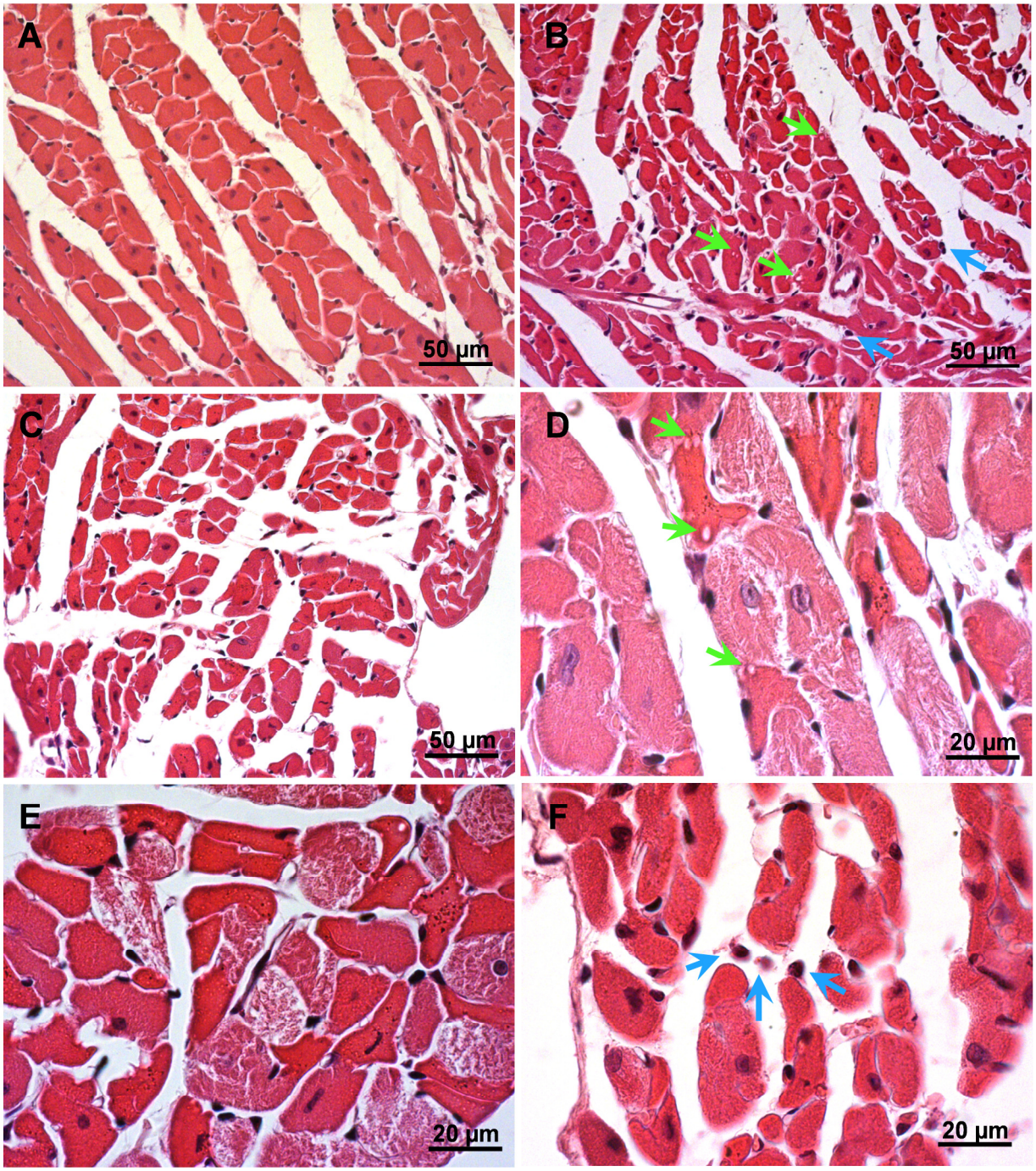

Figure 28 - Cardiac histopathology by light microscopy from MTX-treated animals. (A) Light micrograph from the control of 9.0 mg/kg MTX of adult mice, showing normal morphology and structure; (B) Light micrograph from pediatric mice injected with cumulative dose of 9.0 mg/kg MTX. Vacuolization (green arrow) and inflammatory infiltration (blue arrow) are shown. (C) Light micrograph from pediatric mice injected with cumulative dose of 7.0 mg/kg MTX. This treated group presents large and uncondensed nucleus. (D) Light micrograph from adult mice injected with cumulative dose of 9.0 mg/kg MTX. The cardiomyocytes present high degree of cellular edema with minor structure density, irregular nucleus and vacuolization (green arrow) were observed. (E) Light micrograph from adult mice injected with cumulative dose of 9.0 mg/kg MTX. Cellular edema and necrotic zones are evident. (F) Light micrograph from pediatric mice injected with cumulative dose of 7.0 mg/kg MTX. The inflammatory status of the tissue is clear, as indicated by blue arrows showing the presence of infiltrative inflammatory cells. These results evidence that cardiotoxicity occurred in all MTX-treated animals. Pediatric mice seem to be more protected from damage than adult mice, since pediatric mice had less cardiac histological damage.

5. DISCUSSION

The cumulative dose-dependent cardiotoxicity of MTX remains a major clinical concern. Although MTX shows a clinical profile similar to DOX, their mechanisms of cardiotoxicity differ and MTX-induced cardiotoxicity mechanisms remain largely unknown.

At present, animal models allow to understand the molecular mechanistic basis by which anticancer drugs cause cardiotoxicity, including progressive cardiac dysfunction (Bovelli et al., 2010). However, many studies evaluate the toxic profile of anticancer drugs using acute administration conditions, which does not represent the cancer therapy reality and of the late cardiotoxicity that is the main safety concern of anthracyclines and antracenediones. In fact, their cardiotoxicity might appear as a late event, occurring even years after the completion of therapy (Seiter, 2005). In the present study, a model of multiple MTX administrations was used in male CD-1 mice, to whom was administered MTX via i.p. twice a week for three weeks. Multiple administrations in different time-points were given to mice in order to mimic the human MTX-therapy (Vorobiof et al., 1987, Paul et al., 2007). These multiple administrations aim to gradually elicit a cumulative cardiotoxic dose: the use of an acute high dose would concur to acute MTX-cardiotoxicity that is not the main objective of this dissertation. In accordance, the animals were also maintained for several weeks without further MTX-administrations to allow the development of symptomatology and observe the effects of the previously accumulated MTX. The fast growing animal model used allows the study of several parameters and late effects in a small period of time.

In all the experiments, the MTX administration was given in the afternoon, as it was reported that the three pharmacokinetic half-lives of MTX are shorter in mice in this period of the day, maintaining highest anti-tumor activity with lower toxicity. Thus, this time period was assumed to be more tolerable to MTX administration, mainly when a high-dose delivery is contemplated (Levi et al., 1994).

5.1. General welfare of the MTX-treated animal model

In both experiments, the toxicity of MTX was evident by the observation of the general conditions of the pediatric and adult animals, namely external signs and physiological parameters. In experiment 1, no adult mice survived to the highest dose of 9.0 mg/kg MTX, while in the pediatric population the percentage of survival was 33% at that same high cumulative dose. Adult mice had a significant decrease in body weight, which was not observed in the pediatric groups. A higher toxicity is therefore apparent to the general well-

being of the adult populations. Although the adults of the 7.0 mg/kg group in experiment 2 had decreased body weight, compared to controls, the pediatric animals also showed significant differences in body weight, namely with to less gain of body weight. Altogether, pediatric population appeared to present a higher resistance to the cumulative toxicity-induced by MTX than the adult population. During MTX administrations no changes were observed and these effects on body weight resulted from the MTX total cumulative dose. In fact, in experiment 2, the highest dose of 9.0 mg/kg of MTX did not cause abrupt changes in the weight of the animals sacrificed 24h after the last administration (excepting last administration day, in pediatric mice), in opposition to the 7.0 mg/kg MTX condition, where animals were allowed to develop cumulative toxicity. The loss of body weight in mice has been reported as common for MTX (Levi et al., 1994, Raghunand et al., 2003, Keese et al., 2009, Niang et al., 2011, Yagublu et al., 2013), but so far no data regarding pediatric population existed. Considering the possibility that the significant differences in body weight might be related to differences in food / water consumption, the food and water intakes were recorded twice a week. Normalization to each day and each animal weight was performed, assuming that each animal ate in a proportional manner relatively to its body weight. Comparing to the saline-treated controls, consumptions from MTX-treated animals were not very consistent during the time of the experiments. In general, food and water intake were markedly reduced in the following days after MTX-administration and, together with body weight loss, these data were related to the morbidity caused by MTX. An apparent exception occurred in the 7.0 mg/kg MTX-treated adults, which had higher food consumptions in the week after the last injection accompanied with decrease body weight. However, loss of body weight might not be exclusively due to lower food consumption. Gastrointestinal disturbances have been reported in patients as a common complication of cytotoxic cancer chemotherapy, specifically systemic treatments, as cytotoxic agents do not distinguish between cancer cells and normal cells undergoing rapid division like the ones that exist in the gastrointestinal tract (Di Fiore and Van Cutsem, 2009, Boussios et al., 2012). Therefore, poor absorption may have occurred as a result of the damaging effects of MTX on the epithelial lining of the intestine and, thus, contributed to a significant reduction in body weight in MTX-treated mice. Energy imbalance due to metabolic changes, involving factors such as reduced food intake (imbalances between orexigenic and anorexigenic signals), physical activity (decreased muscle mass and function) and resting energy expenditure are common in chemotherapy regimens (Gadea et al., 2012, Nicolini et al., 2013). Moreover, younger age may favor weight gain (Gadea et al., 2012), which is in concordance to the present study.

The overall condition was also compromised in MTX-treated animals. The necropsy made to the animals that did not survive in experiment 1 showed damage in various organs. Liver presented abnormal external signs of damage, presenting in some cases dark color

while blue stains were common in the peritoneum of MTX-treated animals. Signs of injury were also present in the remaining pediatric animal survivors in the highest 9.0 mg/kg MTX in experiment 1 and in the 7.0 mg/kg MTX dose, namely inertia and absence of diary hygiene, demonstrating that MTX has a time-dependent late toxic profile, an observation that was possible because these groups were not sacrificed immediately after the last MTX administration.

5.2. Biochemical blood analysis and organ damage

Several parameters were determined to assess MTX-induced toxicity in the heart and other organs and whether the different aged populations present different susceptibilities. Plasma AST, ALT, and CK-MB levels were measured in both studies (in experiment 2, total-CK was also measured). Both AST and ALT are present within the hepatocytes and are released into the blood stream when the membranes of hepatic cells are damaged. AST and ALT increase in plasma can result of inflammatory processes, being also sensitive indicators of necrotic hepatic lesions (Rej, 1989, Duong and Loh, 2006). ALT is a more sensitive and specific for liver damage, namely to monitor the effects of hepatotoxic drugs (Wilson, 2007), whereas AST is found predominately in tissues of high metabolic activity, such as heart, skeletal muscle, kidneys, as well as liver (Rej, 1989, Duong and Loh, 2006, Wilson, 2007). Although MTX-treated patients have been documented with transient elevations in plasma AST and ALT levels (Paciucci and Sklarin, 1986), the AST and ALT levels in experiment 1 were decreased in the 4.5 mg/kg MTX-treated adults. Biological explanations to decreased blood aminotransferase activities are difficult to characterize and may be caused by drug-induced metabolic impairment, such as vitamin B_6 (a cofactor in aminotransferase reactions) or zinc deficiency (Waner and Nyska, 1991). These decreased levels are likely associated with metabolic conditions, such anorexia and severe weight loss (Waner and Nyska, 1991). MTX may be indirectly associated with this decrease in aminotransferase activities, since severe loss of body weight due to toxic effect of drugs may possibly lead to the reduction in their activities. Moreover, the gastrointestinal disturbances caused by MTX may contribute to a malabsorption syndrome that decreases the availability of the cofactors required for the activity of the aminotransferases (Di Fiore and Van Cutsem, 2009). Renal failure can also cause decrease aminotransferase activities. Patients with chronic renal failure have shown low plasma AST and ALT that may be or not related to deficiency in vitamin B_6 (Warnock et al., 1974, Waner and Nyska, 1991, Yasuda et al., 1995). Other reasonable explanations may be: suppression of AST and ALT synthesis in the hepatocyte, inhibition of their release from

the hepatocyte into circulating blood, or their accelerated clearance from serum (Yasuda et al., 1995).

Although AST cannot be considered a specific marker for myocardial damage, myocardial muscle is the highest source of plasma AST (Rej, 1989). In experiment 1, the two surviving 9.0 mg/kg MTX-treated pediatrics showed increased plasma AST levels, demonstrating that this population suffered heart damage resulting from MTX-administration. As the ALT levels in this group are not increased, it substantiates that the contribution for elevated AST levels results mainly of the heart. Both ALT and AST are released upon hepatic necrosis, although AST is more slowly released in comparison to ALT (Niang et al., 2011). An increased AST/ALT ratio has been proposed as a method for assessment of heart damage (Rej, 1989, Sánchez et al., 2002). An increased AST/ALT ratio occurred in 9.0 mg/kg MTX-treated pediatrics, thus confirming heart damage. AST/ALT ratio is elevated in acute cardiac damage, however, this ratio tends to decrease after a few days because of a faster decline in AST than ALT (AST persists for 4-6 days after myocardial infarction), which makes this situation sometimes misleading to the prognosis (Nathwani et al., 2005, Duong and Loh, 2006, Wilson, 2007). In experiment 2, no significant differences in the aminotransferase levels were found in any of the MTX-treated groups. The group of 7.0 mg/kg MTX-treated animals showed a significant decrease in the percentage of liver weight / body weight ratio. Significant decreases in relative liver weight in mice with solid form of Ehrlich tumor treated acutely with MTX (6, 9 or 12 mg/kg) were reported, accompanied with increased AST activity in plasma (Niang et al., 2011). However, no increases in AST levels were observed in the 7.0 mg/kg MTX-treated adult mice that a had significant decrease in liver weight / body weight ratio. All these facts suggest that assessment of cardiotoxicity and/or hepatotoxicity by measurement of aminotransferases is not easy to interpret.

CK catalyzes the reversible transfer of the phosphoryl group from phosphocreatine to adenosine diphosphate (ADP) to regenerate ATP. It is located in tissues that have high energy demands in order to contribute to the cells' homeostasis (Wallimann et al., 1998). The myocardial isoenzyme CK-MB is a cardiospecific biomarker that represents up to 30% of total CK in heart and is released from injured cardiomyocytes (Adams et al., 1993, Lewandrowski et al., 2002, Horacek et al., 2007, O'Brien, 2008). In opposition to experiment 1, differences in CK-MB levels were found in the animals of experiment 2. The 7.0 mg/kg cumulative MTX dose induced an elevation in the plasma CK-MB values in pediatric mice, which may be indicative of cardiac damage. In 9.0 mg/kg MTX-treated pediatric mice sacrificed 24h after the last administration, the plasma CK-MB decreased, which may be a result of altered cardiac turnover. Measuring and analyzing the plasma CK-MB levels must be done with caution, since after initial heart injury, CK-MB rise occurs within 4h to 9h after the cardiac onset event, peaks at 24h, and returns to baseline at 48h to 72h (Lewandrowski

et al., 2002). Thus, plasma CK-MB values are highly dependent on the time-point of measurements. In the case of myocardial infarction, multiple determinations and monitoring of CK-MB are recommended (St Louis and Gandhi, 1994). The rapid clearance of CK-MB from plasma makes a particular good diagnosis parameter and perhaps the only advantage over markers that remain elevated for longer periods (like troponins) (Lewandrowski et al., 2002), since it detects recent. Moreover, it was also reported that the diagnostic value of CK-MB in pediatric cases is problematic, since the elevated CK-MB occurred in 8 of 27 patients at risk for cardiac contusion but it was poorly correlated to other tests, including ECGs and echocardiograms (Langer et al., 1989).

The decrease in CK-MB plasma values in 9.0 mg/kg MTX-treated mice is probably a result of cardiac alteration, since CK-MB is produced primarily by heart. Phosphocreatine levels may contribute to the elucidation of this data. However, attention is required when analyzing CK-MB data, since CK-MB accounts approximately for only <3% of the total-CK in the entire organism (Wilson, 2007) and up to 30% of the total-CK in the heart (Adams et al., 1993).

The total-CK values of MTX-animals did not significantly differ from controls in this study. Total-CK is mainly due to skeletal muscle, and CK-MB represents only 3% of total-CK in this tissue (Adams et al., 1993). A case was reported of a patient with cardiac contusion presenting an early and prolonged elevation of CK-MB with normal values of total-CK (St Louis and Gandhi, 1994). Therefore, the overall muscle activity apparently is unchanged by MTX.

5.3. Oxidative stress mechanisms related to MTX

Anthracyclines' toxicity is often attributed to oxidative stress. To determine if MTX caused oxidative stress in the organs evaluated, lipid peroxidation, GSHt and GSSG levels were determined.

In order to assess if MTX caused lipid peroxidation in pediatric and adult mice, the levels of MDA (or TBARS) were determined. Lipid peroxidation is a free-radical-mediated chain of reactions that causes oxidative damage to polyunsaturated lipids, namely components of cell membranes (Grotto et al., 2009). To assess if MTX is able to initiate or enhance lipid peroxidation, by formation of reactive species or by eliciting oxidative stress, the most known secondary product of lipid peroxidation, MDA, was determined. The indirect determination of MDA through the TBARS technique has the pitfall that TBA reacts with a variety of non-MDA compounds, such as sugars, amino acids, proteins, and other non-lipids materials that are present in biological samples (Knight et al., 1988). Although fluorescence is more specific

and sensitive than the similar chromogenic technique, as it avoids interferences of other colorimetric compounds formed, the measurements are still overestimated with this technique. Therefore, in experiment 2, the MDA determination was complemented with the highly specific HPLC technique. In all the experiments and techniques (HPLC and fluorescence), high centrifugation of the samples was done in order to remove all possible contaminants that might interfere with the measurement of MDA. Therefore, free-MDA (unbound to proteins) was determined. In the two experiments, the quantification of free-MDA levels in the heart was not possible. Indeed, biological samples may have low amounts of free-MDA, often close to the detection limit of the method used, while significant amount of MDA is bound to tissue matrix (Pilz et al., 2000). However, in another study, decreased cardiac levels of lipid peroxidation were observed in mice when MTX was administered acutely (15 mg/kg) (Arnaiz and Llesuy, 1993). Nonetheless, the results differ between liver and heart (Llesuy and Arnaiz, 1990). In the liver and kidneys, MDA levels were measurable. MTX caused a significant decrease in MDA levels in the liver of 9.0 mg/kg MTX-treated pediatric mice in experiment 2. The decreased levels of MDA found in this group of animals reveal that MTX does not elicit lipid peroxidation. In several models, MTX was shown to have negative results upon lipid peroxidation or was even able to decrease it. In liver microsomes incubated with 30 µM of MTX, no alterations were observed in lipid peroxidation (Vile and Winterbourn, 1989), while Novak and Kharasch demonstrated an antioxidant inhibitory effect in lipid peroxidation in cardiac mitochondria and sarcosomes, and in liver microsomes treated with MTX (Kharasch and Novak, 1983, Novak and Kharasch, 1985). In opposition in another study, MTX caused increased MDA liver levels increase (Llesuy and Arnaiz, 1990). The administration of MTX to mice in a single dose of 15 mg/kg body weight (i.p.) and the determination of lipid peroxidation 3, 4 and 5 days after injection, showed that MTX caused increases of 73% and 52% in MDA levels and hydroperoxide-initiated chemiluminescence in liver homogenate in this acute administration setting (Llesuy and Arnaiz, 1990).

To further evaluate the redox status of the animals, the GSHt and GSSG levels were determined. GSH is the most important non-protein thiol of the cells and acts as a redox buffer (Kretzschmar, 1996). It carries several essential functions related to the organism's protection (Owen and Butterfield, 2010). GSH acts as an intracellular reducing agent, ROS scavenger and inhibitor of lipid peroxidation (Owen and Butterfield, 2010, Zitka et al., 2012). Its' antioxidant activity occurs through its direct binding with electrophiles, with or without the catalysis of glutathione S-transferases, or through the formation of the dimeric GSSG, an oxidized form of glutathione, as it occurs for the elimination of peroxides (Costa et al., 2011).

In experiment 1, MTX caused significant alterations in the cardiac GSH/GSSG ratio. The GSH/GSSG ratio is an indicator of the redox status of the cells or tissues (Wu et al., 2004, Owen and Butterfield, 2010, Zitka et al., 2012). The 9.0 mg/kg MTX-treated pediatric mice

presented higher values of cardiac GSHt (although not statistically significant), with no increase in GSSG values compared to other groups, thus reflecting in a higher GSH/GSSG ratio. This adaptation response might be related to an up-regulation of γ-glutamylcysteinesynthase (γ-GCS) activity. γ-GCS is responsible for the synthesis *de novo* of GSH (Wu et al., 2004), and its increased activity mainly results as a compensatory response to a drug-induced GSH depletion. In order to maintain GSH homeostasis, the cells increase its synthesis, since GSH acts not only as a cellular protectant but also as regulator of cellular signaling (Tew, 1994, Kitteringham et al., 2000). In fact, the maintenance of cellular glutathione status is critical to the cell's function, and is mediated by enzymes of the glutathione cycle (namely glutathione reductase) and also involves transcriptional mechanisms that are redox sensitive, such as Nrf2–Kelch-like ECH-associated protein 1 (Keap1) signaling system (Gounder et al., 2012, Geenen et al., 2013). In the 7.0 mg/kg MTX-treated pediatric mice, the GSH/GSSG was not changed, showing that with that concentration and lower time elapsed, the heart was not able to yet adapt. The 9.0 mg/kg MTX-treated adult animals, in experiment 2, had higher GSSG values showing that even if MTX has a low potential to cause oxidative stress, by futile redox cycle of the molecule, it interferes with the redox status of the heart. In fact, other authors reported no significant changes in the cardiac GSH levels in mice treated with an acute dose of 15 mg/kg of MTX (Arnaiz and Llesuy, 1993). In the heart of rats treated with DOX, the ratio GSH/GSSG decreased indicating oxidative stress, with increase in GSSG levels (Agapito et al., 2001), while in the present work these events did not happened (at least simultaneously). This further corroborates the different mechanisms of MTX and DOX induced cardiotoxicity.

Regarding hepatocellular damage, in experiment 1, the 9.0 mg/kg MTX-treated pediatric group presented low GSH/GSSG ratio, oppositely to its higher GSH/GSSG ratio in heart evidencing that both organs show dissimilar characteristics towards MTX. In the same experiment, the adult mice (lowest 4.5 mg/kg MTX dose) presented an increased ratio of GSH/GSSG suggesting that the adult mice might have mechanisms to compensate the MTX-induced damage in liver at lower concentrations, while at higher concentrations a true oxidative stress phenomenon is installed. In fact, in this low cumulative concentration (4.5 mg/kg), the hepatic oxidative related damage in adult population is the only change observed. In a rat model, increased hepatic levels of GSHt occurred in a cumulative dose of 7.5 mg/kg-treated rats and sacrificed 28 days after last MTX administration. This change was related not only to an increase in GSH, but also in GSSG (Rossato et al., 2013a). An increased hepatic expression of γ-GCS mRNA and protein, as well nontranscriptional regulation, were reported in CD-1 mice exposed to toxic depleting GSH species, such as buthionine sulphoximine and acetaminophen, as a result of GSH depletion and concerted cellular response to restore homeostasis (Kitteringham et al., 2000). In fact, in experiment 2,

the 7.0 mg/kg MTX-treated adults had a decrease in hepatic GSHt compared to controls, without changes in GSSG, reflecting that MTX had a time-dependent effect upon cellular antioxidant defenses. Decrease in hepatic GSHt values was already reported for MTX. The MTX induced a decrease of 39% in hepatic GSHt values in mice treated with MTX and sacrificed 3-5 days after administration (single dose 15 mg/kg), compared to controls, but the authors did not determine the GSSG content (Llesuy and Arnaiz, 1990), being impossible to infer what other mechanisms are involved. In the model used in this dissertation, MTX alters glutathione pathways in a time- and concentration-dependent manner. The hepatocellular GSH content is not static but the result of a dynamic process, including synthesis by γ-GCS or by the recycling of glutathione reductase, by the utilization, and export rate (Kretzschmar, 1996). Depletion occurred in hepatic GSHt may be associated to drug conjugation or export (Awasthi et al., 1994), or even by diminished nuclear Nrf2 levels (Gounder et al., 2012). MTX metabolism may involve oxidation (Blanz et al., 1991) that can react with GSH, forming conjugates (Rossato et al., 2013b). The irreversible loss of GSH is possibly the consequence of reactions resulting in thiol conjugate formation and efflux from the cell, namely in the form of GSSG or GSH conjugates (Arrick and Nathan, 1984). There is significant evidence that glutathione-xenobiotic conjugates (including the ones formed with DOX) and GSSG are removed from the interior of the cell by ATP-dependent membrane transporters, the multidrug resistance-associated proteins (MRPs) (Homolya et al., 2003, Cole and Deeley, 2006). Moreover, as all MTX-treated groups of experiment 2, with the exception of 7.0 mg/kg MTX-treated pediatric mice, had a significant decrease in hepatic ATP contents, one can consider a possible relationship between the bioenergetics impairment and the glutathione conjugates efflux is possible, as MRP are ATP-dependent. If that hypothesis is confirmed, the presence of MTX can lead to one of the two hits (or both): on one hand, ATP depletion occurred first than GSH decrease and therefore can be the cause for the impairment of conjugate transport by the ATP-dependent transport membrane, favoring the formation of glutathione conjugates within the cell, as corroborated by low GSH values; or ATP depletion could be the consequence of the hydrolysis of ATP by the transporter. Another feasible hypothesis is that GSH synthesis may be depleted as a direct consequence of ATP depletion, since GSH synthesis has two ATP requiring steps (Kretzschmar, 1996).

In the kidneys of MTX-treated animals, the 9.0 mg/kg MTX pediatric group had decreased GSSG levels. GSSG has a nefarious effect in the cells, namely leading to thiol-exchange reactions on thiol residues of proteins (Pompella et al., 2003) and altering their normal functions. The cells have a mechanism to eliminate the GSSG by the glutathione reductase system, thus retrieving GSH (Homolya et al., 2003). In some cases, the activity of the glutathione reductase becomes rate limiting and the alternative for eliminating GSSG is its export (Cole and Deeley, 2006, Costa et al., 2009). The decrease observed may be time-

related to the efflux of GSSG by the MRP family in kidneys, which is common during oxidative stress, translating a protection role to the cells

These results from the redox cellular studies found in this dissertation in pediatric and adult mice correlate with data demonstrating that the overall redox homeostasis decreases with increasing biological age of the mice, either by decreased *de novo* synthesis of GSH or increased oxidation of GSH (Abraham et al., 1978). In fact, declines in cardiac GSH levels with aging seem to be related to the decline of myocardial Nfr2 expression, leading to impaired redox homeostasis (Gounder et al., 2012). The lower content in GSH may have an outstanding effect on the detoxification ability of an senescent organism, providing a toxicological basis for aging (Hazelton and Lang, 1980). Thus, these hypotheses may explain the differences between the pediatric and adult population in handling with oxidative stress, since pediatric mice seem to be in an advantage to face the MTX-induced injuries.

5.4. Energetic (im)balance caused by MTX

As the requirement of chemical energy in the form of ATP, to support systolic and diastolic work of the heart, is absolute and ATP levels are approximately decreased in 25% to 30% in the failing human heart (Ingwall and Weiss, 2004), it was evaluated if MTX had effects in the bioenergetics of the animals in experiment 2. MTX has been described to cause depletion of ATP in cardiomyocyte cultures (86% to control) (Neri et al., 1984) and also in neonatal rat heart myocytes in a concentration- and time-dependent manner (Shipp et al., 1993). Recently, a depletion of ATP levels around 50% in H9c2 cells treated with MTX was reported (Rossato et al., 2013c). However, in the present dissertation, no changes in cardiac ATP were verified in the heart of MTX-treated mice when compared to control animals. Probably, the animal sacrifice occurred before cardiac failure was installed. Although few data is available on the effect of MTX in bioenergetics in live systems, some studies involve MTX administration in an acute form (Llesuy and Arnaiz, 1990, Arnaiz and Llesuy, 1993), thus misleading the nature of the possible mechanisms by which MTX actually provokes toxicity. The primary energy reserve in the heart is phosphocreatine and the enzyme CK transfers the phosphoryl group between ATP and phosphocreatine much faster than the rate of ATP synthesis by oxidative phosphorylation (Ingwall and Weiss, 2004). In fact, heart failure is associated with decreased CK activity (Saupe et al., 1998). Under conditions when ATP demand is higher than ATP supply, as in case of cardiac failure, the use of phosphocreatine via the CK system is crucial for the heart maintenance of constant ATP levels (Ingwall and Weiss, 2004). Thus, in the future, determinations of the levels of phosphocreatine might answer and clarify if the energy status of the animals is

compromised. Moreover, it is possible that other sources of energy allow the maintenance of ATP levels and only at the end-stage heart failure, the bioenergetics of the heart fails completely. However and although CK-MB represents up to 30% of the total-CK in heart (Adams et al., 1993), the decrease in plasma CK-MB levels may be correlated with an early impairment in the energetic balance of the heart.

Hepatocellular bioenergetics was significantly impaired in MTX-treated mice. An approximately 3-fold and 2-fold decrease in ATP levels occurred in adult mice at 9.0 mg/kg MTX and 7.0 mg/kg MTX, respectively. In pediatric mice, decrease in ATP values also occurred, but only in the 9.0 mg/kg MTX-treated group. Depletion of ATP, together with oxidative stress, is one of the most common causes of liver damage (Bantel and Schulze-Osthoff, 2012). Necrosis is typically characterized and accompanied by severe ATP depletion, whereas apoptosis is an ATP-dependent cell death program (Ferrari et al., 1998). Thus, depletion in ATP reserves in all MTX-treated groups, (with the exception of the 7.0 mg/kg MTX-treated pediatric mice), could in some way be an indicator of cell death by necrosis in liver, as already demonstrated by Ehninger *et al.* in isolated perfused rat liver (Ehninger et al., 1984). However, a same drug can induce cell death by necrosis depending of the dose and time of administration (Bantel and Schulze-Osthoff, 2012) and ALT values showed no differences in these animals, thus not confirming the MTX-induced necrosis in the liver. The ATP depletion may be related to other phenomena, namely MTX metabolism. It is described that MTX suffers metabolism in liver (Ehninger et al., 1984, Ehninger et al., 1990, Mewes et al., 1993). The complete loss of the cytotoxicity of MTX in HepG2 cells when cytochrome P-450 is inhibited, suggests that MTX is cytotoxic when it is metabolized and oxidized in the liver (Mewes et al., 1993). The liver is the most important organ of metabolization and any xenobiotic that suffers metabolic metabolization can be a potential hepatotoxic. MTX has been shown to be bioactivated by hepatic cytochrome P-450 super family (Blanz et al., 1991, Mewes et al., 1993). The metabolites formed, namely naphthoquinoxaline cause ATP depletion in cardiac cell models (Shipp et al., 1993), however those data are not yet confirmed in hepatic cellular models.

In rats, the administration of a cumulative dose of 7.5 mg/kg of MTX and sacrificed 22 days after the last MTX administration leads to decrease in hepatic ATP levels (Rossato et al., 2013a). Even so, no further mechanisms were exploited. The determination of ROS and calcium levels might help to further understand the responsible mechanisms for depletion of ATP, as they are responsible for membrane mitochondrial disruptions and ATP production impairment (Brookes et al., 2004).

5.5. Lymphocytes and MTX

MTX has known immunosuppressant activity (Fox, 2004). In experiment 2, the 9.0 mg/kg MTX-treated pediatrics and adults presented a decline in lymphocyte number. These facts suggest that the highest dose of 9.0 mg/kg has a high impact in the immune system of the animals. In a study in MTX-treated mice with cumulative dose of 7.0 mg/kg for 14 days, MTX exerted a suppressive influence on the humoral immune system (Fidler et al., 1986). Low lymphocyte levels in rats treated with a cumulative dose of 7.5 mg/kg of MTX and sacrificed 2 days after the last administration were previously reported (Rossato et al., 2013c). No major inferences can be done between pediatrics and adults about lymphocytes and the effect of MTX in these populations since data were scarce as only representative animals were taken.

5.6. Histopathological examination of cardiac damage

Histopathological changes in the heart evidenced the toxic profile of MTX in experiment 2. Both MTX-treated pediatric and adult mice showed inflammatory activity by the presence of mild leukocyte infiltration (1 to 3 cells by visual field) in the interstitial space of cardiomyocytes. Indeed, myocardial inflammation is a common consequence of myocardial injury (González et al., 2011). Evaluating cellular degeneration, adult mice showed higher degree of damage compared to pediatric mice, showing a greater extent of cellular vacuolization and cellular edema, as well as some sporadic zones of necrosis. The pediatrics group treated with 9.0 mg/kg MTX dose, although sacrificed one day after the last MTX administration, presented higher damage regarding to necrosis events (statistically different) compared to it control group, than animals administered 7.0 mg/kg MTX, evidencing that necrosis may be occurring in a dose-dependent manner. Vacuolization events were also described, by transmission electron microscopy, in MTX-treated myocytes from neonatal rats after 2 µm/mL exposure to MTX (Shipp et al., 1993), and in a endomyocardial biopsy section from a patient treated with 204 mg/m^2 of MTX (Aapro et al., 1983). In dogs that received 6 courses of 0.25 mg/kg of MTX after 7 weeks of initial DOX treatment (4 courses of 1.64 mg/kg DOX), moderate myocytic vacuolization, as well as occasionally interstitial edema were seen (Tham et al., 1987). Vacuolization was also seen in anthracyclines-based regimen in the heart of patients studied at necropsy (Isner et al., 1983) and in mice (Rahman et al., 1982, Desai et al., 2013), as well inflammatory infiltration, edema and necrosis (Dudka et al., 2012), thus demonstration that histological damage is similar in anthracyclines and MTX, while the involved mechanisms seem to differ. Comparing necrosis to CK-MB or AST levels,

the results are contradictory to what was discussed in the CK-MB section, which make us to conclude that it is necessary to carefully analyze the data, since a correlation between necrosis and CK-MB or AST is not always valid, depending on the extent of the injury and the elapsed time since the damage occurred. As CK-MB peaks in plasma at early 24h after cardiac injury and rapid decline (Jaffe et al., 2006), it is reasonable to conclude that CK-MB is not a good predictor of cardiac injury in cases of very early or late cumulative cardiotoxicity. Indeed, significant elevations of troponins are thought to better reflect myocardial necrosis, as patients with detectable troponins, but no plasma CK-MB, may reveal microscopic myocardial necrosis events (Lewandrowski et al., 2002). A way to solve this question is to analyze the cardiac tissues by immunohistochemistry with specific markers of necrotic cells.

In the semi-quantitative histological evaluation, pediatric mice appear to be more protected than adult mice, since necrosis and cellular degeneration occurred at a lower extent than in adult mice, as demonstrated by the scores. Likewise, the cardiomyocytes of pediatric mice sporadically showed large nuclei and more than one nucleolus, which might be indicative of higher nuclear activity for synthesizing protein products or other cellular metabolism. The pediatric population had a better conserved periphery than adults.

It was not possible to distinguish whether MTX provoked cardiotoxicity in a concentration-dependent manner because the groups that received the two cumulative doses were sacrificed at a different time-point and MTX causes cumulative damage. Thus, the assessment of damage would be erroneously rated. However, a striking evidence was observed: the animals of 9.0 mg/kg MTX, namely adults, developed a greater cardiac damage in the endocardium zone, while the damage in 7.0 mg/kg MTX animals was significant in endocardium but towards to pericardium zone. This effect demonstrates that the MTX causes toxicity not only during the treatment, but also after its administration, corroborating the characteristic of chronic cardiotoxicity in this type of anticancer therapy and its large accumulation in the heart (Ehninger et al., 1990). Other evaluations in these groups of animals have to be made, to better understand what mechanisms are implicated in the cardiotoxicity observed, such as the evaluation of conjunctive tissue or specific markers by immunohistochemistry, like damage in progenitor cells.

5.7. Apoptosis in the heart

Caspases are main components in the mechanisms responsible for apoptosis, representing a regulated molecular process that removes excess or unwanted cells by controlled autodigestion from organism, and can be activated by exogenous stimuli such as

inflammation, hypoxia, radiation, and chemotherapeutic drugs (Thompson, 1995, Shi, 2002). Therefore, the possibility of cell death by apoptosis was assessed in experiment 2, measuring caspase activities: the initiators caspase-8 and caspase-9, and the downstream effector caspase-3. MTX did not promote increase in the caspase activities in the MTX-treated groups. In fact, a decrease in caspase-3 activity in 9.0 mg/kg MTX-treated pediatric animals was observed. This result has to be further investigated, since it was already shown that MTX activates caspase-3 in H9c2 cells incubated with 100nM and 1µM MTX (Rossato et al., 2013c) and with 1.60 µM MTX (Kluza et al., 2004), although this fact was only demonstrated in *in vitro* cellular models. Some studies reported decreased caspase-3 activities when exposed to oxidative environment, such superoxide and $^{\bullet}$NO. High concentrations of $^{\bullet}$NO are proapoptotic, whereas low concentrations have been shown to be protective against apoptosis (Jiang et al., 2009). The mechanism by which apoptosis is decreased could to be related to the S-nitrosylation of caspase-3, as demonstrated in primary cultures of fibroblasts from a patient treated with peroxynitrite (Jiang et al., 2009) and in neonatal cardiomyocytes from rats treated with DOX (previously treated with $^{\bullet}$NO) (Maejima et al., 2005). Thus, further investigation (namely protein nitration data) regarding this matter is need be fully understand the mechanisms implicated in the caspase-3 activity inhibition verified in the 9.0 mg/kg MTX-treated pediatric mice and whether $^{\bullet}$NO is involved in this issue or if the redox state, MTX concentration, exposure time and combination with oxygen, superoxide and other molecules in this group are involved in rescuing cardiomyocytes from apoptosis (Maejima et al., 2005).

6. CONCLUSIONS

The main objective of this dissertation was to assess if pediatric population was more resistant or prone to MTX toxicity. The general welfare of the animals showed that the pediatric population is more resilient to MTX-induced toxicity as, in experiment 1, some of these animals survived with the 9.0 mg/kg MTX cumulative dose. In both experiments, the average body weight of pediatric mice almost did not vary when compared to controls and, when changes occurred, they were lower when compared to adult mice. Assessment of plasma aminotransferases, as well as the cardiac marker CK-MB revealed different results in the MTX-treated animals compared to controls. At a first sight, they seemed sensitive indicators of the MTX-induced damage. Elevations of AST in 9.0 mg/kg MTX-treated pediatric animals in experiment 1 may be the result of heart damage since no changes in ALT levels were seen. The decreases in AST and ALT in 4.5 mg/kg MTX-treated adults may be related to their body weight decreases or to metabolic changes. Regarding to the data obtained in plasma CK-MB levels in pediatric mice, the increase observed in 7.0 mg/kg treated group and the decrease in the 9.0 mg/kg treated group revealed that this parameter is time- and injury-dependent.

Lipid peroxidation was not altered in any groups and organs, excepting in the liver of 9.0 mg/kg MTX-treated pediatric mice in experiment 2, in which lipid peroxidation was decreased. MTX is known to have low ability to cause lipid peroxidation. In experiment 1, the evaluation of glutathione in the heart showed that 9.0 mg/kg MTX-treated pediatric mice had increased GSH/GSSG ratio, indicating that they may have adapted increasing their antioxidant defenses over time towards MTX-induced toxicity. Oppositely, the 9.0 mg/kg MTX-treated adults, which were not allowed to develop cumulative toxicity (experiment 2), showed higher values of cardiac GSSG. Herein, we conclude that pediatric mice developed with the elapsed time compensatory mechanisms against the cardiotoxic potential of MTX increasing their cardiac GSH levels. The heart and liver showed dissimilar characteristics towards MTX. Adults of 4.5 mg/kg MTX dose had an increased hepatic GSH/GSSG ratio, while the 7.0 mg/kg MTX dose had a decrease in GSHt values. These data suggest that adults developed mechanisms in the liver to compensate the damage induced by MTX, through GSH synthesis or by drug conjugation/export. The liver of the 9.0 mg/kg MTX-treated pediatrics showed low GSH/GSSG ratio in experiment 1, not revealing this adaptation mechanism. The lower dose of 7.0 mg/kg in adults showed similar results in the liver of the 9.0 mg/kg-surviving pediatric animals regarding GSH/GSSG, revealing that adults are more sensitive to the toxicity of MTX. These results of oxidative stress suggest that biological age of the mice has influence in the redox defense potential and detoxification.

Hepatic energetic impairment occurred in all MTX-treated animals in experiment 2, excepting in 7.0 mg/kg MTX-treated pediatric mice, which proves that this younger population is more resilient to MTX-induced toxicity. Furthermore, this conclusion was corroborated in the histological study of the heart: pediatric population had less cardiac damage. Both populations showed inflammatory activity, cellular degeneration, with cellular edema and vacuolization, as well as some sporadic zones of necrosis, however in a lower degree in pediatric mice.

Time is a major handicap to appraise the mechanisms involved in the cardiotoxicity of MTX, since the animals may have develop time-dependent compensatory mechanisms and, thus, give different results of a same parameter, depending of the elapsed time. Investigation should be made comparing several parameters, doses and time, allowing understanding and corroborating the different mechanisms associated to the observed toxicity. The results presented demonstrate that evaluating a toxic profile of a drug in a living system requires a multifactorial analysis.

Elapsed time after MTX administration and cumulative dose are the main factors to consider when studying MTX toxicity, but the age of the animal models should also be taken into account. More time should be given to assess whether the late cardiotoxicity would develop in non-lethal doses. Moreover, the determination of phosphocreatine levels and the evaluation of fibrotic tissue in the heart will allow a better understanding of the real cardiac status, as a functionally working myocardium is crucial. The evaluation of progenitor cardiac cells in either adult or pediatric populations would also allow assessing if those populations have different susceptibilities that could lead to late cardiotoxicity.

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